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Department of Pharmacognosy

Ph.D. Thesis

Serratula wolffii, as a promising source of ecdysteroids

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Abbreviations

^{13}C NMR = carbon nuclear magnetic resonance spectroscopy

1D = one dimensional

2D = two dimensional

^{1}H NMR = proton nuclear magnetic resonance spectroscopy

20E = 20-hydroxyecdysone

CC = column chromatography

CIMS = chemical ionisation mass spectroscopy

CN = cyano silica

COSY = correlation spectroscopy

DEI = desorption/electrospray ionization mass spectroscopy

DEPT = distorsionless enhancement by polarization transfer

E = ecdysone

EIMS = electron-impact mass spectroscopy

ESIMS = electrospray ionization mass spectroscopy

FABMS = fast atom bombing mass spectroscopy

FT-IR = Fourier-transformation infrared spectroscopy

HMBC = heteronuclear multiple bond coherence spectroscopy

HMQC = heteronuclear multiple quantum coherence spectroscopy

HPLC = high performance liquid chromatography

HRESIMS = high resolution electospray ionization mass spectroscopy

IR = infrared spectroscopy

MP = mobile phase

MS = mass spectroscopy

NOE = nuclear Overhauser-effect

NOESY = nuclear Overhauser-effect enhancement spectroscopy

NP = normal-phase

OPLC = overpressured layer-chromatography

pB = polypodine B

RP = reversed-phase

SP = stationary phase

TLC = thin layer chromatography

TOCSY = total correlated spectroscopy

UV = ultraviolet

XX = repeated crystallization

1. Introduction

1.1. Ecdysteroids - occurrence and chemistry

Steroid hormones are widespread in nature. Not only the known steroids of the vertebrates, but, for example, the brassinolids, which are plant growth hormones, have a steroidal skeleton. The steroidal hormones of the invertebrates are the ecdysteroids [**1**,**2**]. The first discovered ecdysteroid was ecdysone (**Fig. 1**), which was isolated from silkworm pupae by Butenandt and Karlson in 1954 [**3**]; its structure was elucidated by Huber and Hoppe [**4**].

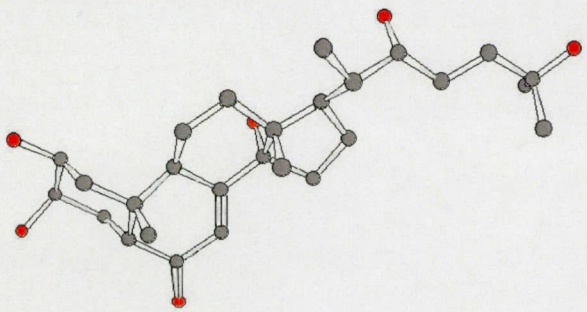


Fig. 1. Steric structure of ecdysone

Ecdysteroids are responsible for the regulation of moulting and the control of embryogenesis and vitellogenesis [**5**-**7**]. They are toxic to arthropods; the application of exogenous ecdysteroids to insects can make their development irregular, resulting in abnormal forms [**8**-**10**]. Ecdysteroids also occur in other invertebrates, such as helminths, corals, molluscs and echinoderms [**8**,**11**,**12**], but further studies are required to elucidate their role in these species.

The compounds structurally related to the insect moulting hormones were subsequently discovered in the plant kingdom. It has been found, that certain plants are rich sources of ecdysteroids, often biosynthesizing these compounds in much larger amounts than arthropods (50 ng/g-30 mg/g) [**1**,**13**]. The ecdysteroids definitely play a defensive role against non-adapted herbivores. However, insects are not able to biosynthesize the steroidal structure *de novo* [**14**]; under certain circumstances, some dietary ecdysteroids can even be beneficial to them. An interesting fact relating to this question is that *Morus alba*, the obligate nutrition of the monophagous silkworm (*Bombyx mori*), is also an ecdysteroid-synthesizing plant [**15**].

Structurally, the ecdysteroids are a well-defined, special group of steroids [**8**,**16**]. They usually contain a C_8-C_{10} alkyl or alkenyl side-chain at position C-17 (C_{27}-C_{29} ecdysteroids), similarly to sterines and brassinolides (**Fig. 2**).

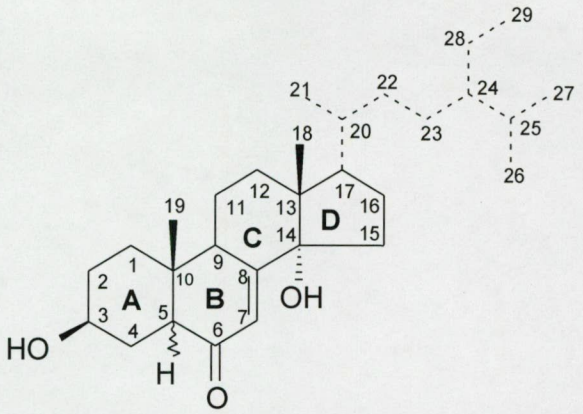


Figure 2. General skeleton of ecdysteroids

The side-chain often forms a ring as a cyclic ether or ester (lactone). Cleavage of the side-chain leads to C_{19}, C_{21} and C_{24} ecdysteroid derivatives. C_{19} and C_{21} ecdysteroids are substituted with a hydroxy, carbonyl or acetyl group at position 17. There is only one known C_{24} ecdysteroid, which has a five-membered lactone ring on C-17. In the typical structure of ecdysteroids, there is an α-OH group at position 14, a β-OH at position 3 and a 7-en-6-one chromophore on ring B. The A/B ring junction is *cis* and the C/D is *trans*. They are usually 5β-androstane steroids with multiple hydroxylation (3-8 OH groups). They occur in nature as acetonides, or esters with organic (acetic, benzoic, coumaric, cinnamic or pyrrole-2-carboxylic acid) [**17-19**] or inorganic (phosphoric or sulfuric) acids. The presence of other olefin bindings and/or carbonyl groups may further elevate their structural variability [**20**,**21**].

1.2. Effects of ecdysteroids

In view of the suggested defensive role of ecdysteroids in plants, these compounds seemed to be an extremely favourable candidate group of natural pesticides. However, it was soon found, that, because of their relative high polarity, ecdysteroids can not penetrate the exoskeleton of insects, and therefore they can not be applied as a spray. Accordingly, their synthetic functional analogues, bisacyl-hydrazines (*e.g.* tebufenozide and RH-2485) are mainly of interest in respect of pest control [**22**]. Nevertheless, investigations of the insect toxicity of ecdysteroids can result in the discovery of new structure-activity relationships, which can form a good starting-point for the design new selective pesticides. Another interesting use of ecdysteroids and/or ecdysteroid analogues can be their application in the use of gene therapy in the future. Extensive research is in progress to plan various induced gene-expression systems based on modified ecdysteroid receptors [**23**].

Ecdysteroids are structurally quite different from mammalian steroid hormones. This can explain their extremely low toxicity on mammals: in the mouse, the LD_{50} of 20E is 6.4 g/kg for intraperitoneal injection and it is >9 g/kg for oral administration [**24,25**]. On the other hand, they display a wide array of pharmacological effects. The anabolic effects of several phytoecdysteroids (20E, cyasterone and turkesterone) on mice or rats were already reported in the late 1960s [**26-29**], and some structure-activity relationships were also discovered [**30**]. It was also reported, that in spite of their anabolic action, they have neither androgenic nor oestrogenic (or antioestrogenic) effects; they do not induce virilization or significant changes in castrated animals [**30,31**]. A few human trials have been performed in this area [**32,33**]. For example, ecdysten®, a 20E-containing product (5 mg/tablet) is officially available as a tonic in Russia. Ecdysteroids can enhance sexual functions in humans [**34**]. Stimulation of growth in mice, rats, sheep, quail and pigs has also been reported [**35-41**].The promoting effect of topically applied ecdysteroids on cell-proliferation and differentiation makes them appropriate for the treatment of wounds [**42-47**]. Ecdysteroids exhibit analgesic [**48**] and anti-inflammatory [**49**] effects too. In consequence of their antidiabetic effect, in many countries (mainly in the Far East), ecdysteroid-containing medicinal plants and preparations are used (traditionally and also officially) as oral antidiabetic drugs in human therapy [**50,51**].

However, most of the above effects were studied *in vitro* and/or in animals, and their relevance for human therapy is poorly understood. Structure-activity relationships in humans are little known. Nevertheless, numerous ecdysteroid-containing products are already available on the market, generally licensed as dietary supplements, or distributed on various webshops with questionable licensing status [**52**], offered with various indications (primarly as anabolics and performance enhancers). In the absence of properly designed clinical studies, and of the relative efficacy and safety profiles of ecdysteroid-containing preparations, it is difficult today to make any sound judgement concerning the real therapeutic potential of the ecdysteroids. Of special concern are the recommendations for anabolic/body-building purposes in sports.

1.3. *Serratula wolffii* Andrae - occurrence and botanical description

The *Serratula* genus is a member of the *Asteraceae* family, *Tubuliflorae* subfamily, *Cynareae* tribe, *Centaureinae* subtribe. They are perennial plants. Four *Serratula* species occur in the Carpathian basin: *S. tinctoria* L., *S. wolffii* Andrae, *S lycopifolia* L. and *S. radiata* Waldst. *et* Kit. *Serratula wolffii* is endemic in western Siberia, southern Russia, Moldova and Transylvania; the westernmost occurrence of the plant is near Kolozsvár (Cluj

Napoča) in Transylvania [**53**]. The name *wolffii* is due to Gábor Wolff (Kőhalom, 1811-1892, Torda), a Hungarian pharmacist of Torda, who was an inspired researcher of the Transylvanian flora [**54-55**].

The stems of the plant are about 80-150 cm high, stout, erect and subglabrous. The basal leaves are irregularly pinnatifid, while the segments are usually elliptic-lanceolate, and sometimes pinnately lobed to subentire. The leaflets are irregularly serrate (hence the English name sawwort), with setulae on the margin and veins. The cauline leaves are similar, becoming reduced upwards and grading into the bracts. The capitulum is about 25-30 mm, campanulate, with up to 15 in a lax, located as an irregular panicle. The outer involucral bracts are cute, velutinous. The inner bracts are rather rigid, long-attenuate, sometimes slightly geniculate and hooked at the apex. The florets are purple. The plant is gynodiecious [**56**].

Serratula wolffii [**57**]

1.4. Chemical overview of the *Serratula* genus

Inasmuch as the members of the *Tubuliflorae* usually do not contain latex, the *Cynareae* species, including the *Serratula* species, do not either.

The species of the genus are rich in flavonoids. The presence of apiin, apigenin, chrysoeriol, luteolin, luteolin-4'-O-glucopyranoside, luteolin-6-C-glucoside, 6-methoxy-luteolin, quercetin, quercetin-3-methyl ether, quercetin-4'-O-glucopyranoside, kaempferol and kaempferol-3-methyl ether [**58-60**] has been established in various *Serratula* species. Some *Serratula* species contain arbutin, and a few lignans have also been identified [**61-62**]; one of them, arctiin, seems to be a chemotaxonomical marker of the *Cynareae* [**62**]. Like the *Asteraceae* species, *Serratula* species also contain polyacetylenes [**63**]. The occurrence of mono- and diterpenoids, and sesquiterpene lactones with a germacrane or guajane skeletone has been confirmed in the genus [**61**,**64**,**65**], and caryophyllen and its epoxide have also been isolated from *S. wolffii* [**63**].

The *Serratula* species are particularly rich in ecdysteroids. *S. tinctoria* contains 0.19-1.30 % of 20E, while *S. wolffii* was found to contain amounts of 20E between 0.13-0.85 %, by TLC-densitometric determination (**66**). **Table 1** shows the ecdysteroids isolated from various *Serratula* species.

Table 1. Ecdysteroids isolated from *Serratula* species.

Serratula species	Ecdysteroid	Reference
S. algida Iljin.	20E	[76]
S. centauroides L.	20E; viticosterone	[67]
S. chinensis S.Moore	20E	[68]
S. coronata L.	20E; E; ajugasterone C; pB; 3-epi-20E; 20E 22-acetate; 22-desoxy 20E (taxisterone)	[69]
	coronatasterone	[70]
	integristerone A; pterosterone; 20E-20,22-acetonide; 20E-2,3;20,22-diacetonide; ajugasterone C 20,22-acetonide	[74]
	inokosterone; viticosterone E; makisterone A	[71]
	makisterone C; ajugasterone C; dacryhainansterone	[72]
	E 22-acetate; (25S)-inokosterone 26-acetate; 20,22-O-(R-ethylidene)-20E, 20,22-O-(R-ethylidene)-ajugasterone C; 20E 2-acetate; 20E 3-acetate	[73]
S. inermis Gilib.	20E	[67]
	2-desoxy-20E; viticosterone E; pB; integristerone A; 20,26-dihydroxyecdysone	[74]
S. komarovii Iljin.	20E, integristerone A; 2-deoxy-20E	[75]
S. lyratifolia Schrenk.	20E	[76]
S. manshurica Kitag.	20E	[77]
S. procumbens Regel.	20E; viticosterone E	[78]
S. quinquiefolia Bieb.	20E	[78]
S. sogdiana Bunge	20E	[76]
	sogdisterone	[79]
	20E; viticosterone E	[80]
	20E	[81]
	viticosterone E	[82]
S. strangulata Iljin.	(24R)-24-(2-hydroxyethyl)-20E	[83]
	20E; 25-desoxy-11,20-dihydroxy-E	[84]
	20E 20,22-acetonide	[85]
S. tinctoria L.	20E; 20E 2-acetate; 20E 3-acetate; 20E 22-acetate; rubrosterone; poststerone; pB; pterosterone; makisterone C; 20E 2,22-diacetate; 20E 3,22-diacetate; 5β-hydroxyrubrosterone; 3-epi-poststerone; 3-epi-rubrosterone; 22-oxo-20E	[86]
	22-epi-20E; gerardiasterone	[87]
S. wolffii Andrae	20E; pB; pterosterone; integristerone A; 20E 20,22-acetonide; 20E 2,3;20,22-diacetonide; ajugasterone C; ajugasterone C 20,22-acetonide	[88]
S. xeranthemoides Bieb.	20E; integristerone A	[89]

1.5. Screening of plants for ecdysteroids

There are both biological and chromatographic methods with which to detect and determine ecdysteroids [**90-99**]. Off-line (HPLC-RIA-biotest [**90,93**] and TLC-UV spectroscopy [**94**]) and on-line (TLC-MS [**95,96**], GC-MS [**97**], HPLC-MS [**21,98**] and HPLC-NMR [**99**]) methods are also available. There are some multiple on-line methods, the "multi-hyphenated" techniques (HPLC-IR-UV-NMR-MS), but these processes still have serious technical problems [**99**]. Currently, only the known ecdysteroids can be detected with these methods to obtain structural information [**100,101**].

Biotests such as radioimmunoassay (RIA) are also appropriate for the detection of these biologically active compounds, but they do not detect inactive ecdysteroids. Nevertheless, these methods are widely used to establish the presence of ecdysteroids in herbal extracts. Biotests are sensitive, but not selective enough, because compounds with different structures can display effects similar to those of ecdysteroids [**21**].

Among the chromatographic methods, thin-layer chromatography (TLC) is appropriate to map the ecdysteroid profile of herbal extracts and identify its components, particularly if the analyte is rich in ecdysteroids and a high sensitivity is not particularly necessary.

Classical adsorption TLC is the most widely used method [**92,101**], but chemically bonded stationary phases (primarily C_{18}) can also be applied. Mainly dichloromethane or ethylacetate-based solvent systems are used for NP-TLC, whereas in RP-TLC mostly water-methanol solvents are used.

The components can be visualized with UV light at 254 nm using plates containing a fluorescent additive; ecdysteroids appear as dark spots. Another possibility for detection is to spray the plate with a reagent which is capable of dehydration; fluorescence can be observed at 366 nm and the spots can be detected in daylight as well. Vanillin - sulfuric acid is commonly used as reagent.

There has been one publication on the application of OPLC in the screening of ecdysteroids [**102**], but this technique is also promising, with good resolution and reproducibility.

HPLC is a fundamentally important method in research into ecdysteroids. It is widely used because of its high resolution. A wide range of SPs and MPs is used, both on NPs and RPs. In the NP-HPLC of ecdysteroids both adsorbent or polar, chemically bonded, both apolar, chemically bonded SPs are available. Silica, which is the most widely used, yields outstanding resolution in the separation of ecdysteroids, as confirmed by many articles [**21,92,98,103,104**]. The most generally applied MPs on silica are ternary systems based on dichloromethane, with isopropanol as organic modifier. Many problems in the separation can

be solved by utilizing different ratios of these two organic solvents, but, because of the strong ecdysteroid adsorption on silica, the peaks display extensive tailing in this case. To make them approximately symmetrical, water has to be added to the solvent system, but this slows equillibrum formation and gradient elution is not possible. Water adsorbs on the SP, slowly deactivating it, and this results in change in the retention times, thereby reducing the reproduciability of the analysis. Isooctane or cyclohexane-based, alcoholic modifiers and water-containing solvent systems are also used [**21,105**].

Lafont *et al.* [**105**] found that the application of overused apolar chemically bonded SPs (TMS) can lead to the very efficient separation of ecdysteroids. They give shorter retention times and symmetric peaks, besides different selectivity to those on silica columns. Both NP and RP-HPLC can be performed in this way on the same SP, by altering the MPs. The behaviour of TMS SPs depends strongly on the amount of free silanol groups.

In RP-HPLC, C_8 and C_{18} modified phases are widely used [**106**]. Different MPs can greatly affect the selectivity. Methanol-water and acetonitrile-water solvents are generally most appropriate, with somewhat higher resolution in the latter case. The peaks can be made symmetrical by adding a buffer.

For the detection of the ecdysteroids during HPLC analysis, mostly UV light is applied. The 7-en-6-one chromophore has sufficient molar absorbance (ε = 10000–14000, λ_{max} = 236–245 nm) to allow the detection of nanograms of ecdysteroids. With modern diode array detectors, more information can be obtained [**21,98**]. The identification of ecdysteroids can be made more specific if HPLC is connected on-line with MS. HPLC-MS analysis is a very sensitive, selective technique with which to identify known ecdysteroids [**21,103,105,107**], though MS data together with retention times are not sufficient to establish the structure of a new compound. Some HPLC–NMR and HPLC–IR–UV–NMR–MS [**99**] methods are available too, but the results are still ambiguous; their development poses a challenge for the near future.

1.6. Methods in the isolation of ecdysteroids

Two trends are predominating in the strategies of ecdysteroid isolation at present. On the one hand, developments permit lowering of the amount of source sample (< 200 g); on the other hand, the aim is to recognise the secundary metabolites of plants completely, according to the metabolomics program [**108-111**]. Both processes need a well-designed strategy [**112**]. To satisfy the aim of the metabolomics program, a high amount of plant sample must be processed (~ 1 ton). Isolation in such large amounts results in numerous ecdysteroids in

amounts high enough for biological measurements, and at the same time it leads to the discovery of minor components, which were so far undetectable [**113**].

After the extraction, both conventional (liquid-liquid extraction) and modern (solid-phase extraction) methods are in use for the prepurification [**1,2,114**]. In the processing of large amounts of sample, further purification is achieved via repeated column-chromatographical steps on silica, alumina and Sephadex LH_{20} stationary phases [**101,115**]. Final purification can be carried out with HPLC. If the sample is small, only consecutive steps of NP- and RP-HPLC are applied [**116-117**]. SPs for gel chromatography are widely available [**118**], but there is only one published example reporting the successful separation of ecdysteroids in this manner [**113**]. Of the liquid-liquid partition-based chromatographic methods, droplet countercurrent chromatography (DCCC), and rotation locular countercurrent chromatography (RLCC) are of value [**119-122**]. There have also been reports of the application of micellar electrokinetic chromatography [**98**] and supercritical chromatography [**123**]. XAD-2 resin has been used in low pressure liquid chromatography [**115**], but its application has not spread.

1.7. Objectives

As seen above, there is growing interest in raw materials suitable for the economic, large-scale extraction of ecdysteroids, and particularly 20E. The plants used for this purpose are at present not available in this region (*e.g. Achyrantes* and *Cyathula* species), not suitable for extraction, due to their relatively low ecdysteroid content (*Spinacia oleracea* and *Chenopodium album*) or do not produce sufficient biomass per land surface. Alternatively the cultivation of few species native to Hungary which do contain sufficient amounts of 20E (*Silene otites*, *Silene nutans* and *Serratula tinctoria*) does not seem feasible. These facts led us to the investigation of further *Serratula* species. *S. wolffii*, the subject of this dissertation, meets the above criteria: it is a robust perennial plant, which does not have special climatic requirements [**56**]. A survey of the pertinent literature indicates that the ecdysteroid composition of *S. wolffii* has hardly been investigated (see **Table 1**).

The results of the previous screening [**66**] indicated that the plant may contain almost 1% of 20E, and our preliminary TLC experiments on the extract suggested a unique ecdysteroid pattern. Thus, our objectives were as follows:

1. To isolate new natural ecdysteroids from the plant, if possible. This objective also involves identification of the ecdysteroids of *S. wolffii*, which are already known from other species, thereby extending the available knowledge on the species and/or the genus.

2. To isolate biologically active compounds. We decided to take into consideration the known structure-activity relationships, and to find ways to identify the presumably effective compounds prior to their isolation. Inasmuch as this objective can be fulfilled, the isolation procedure may be aimed at these ecdysteroids.

3. In view of the highly complex pattern of the minor ecdysteroids in the crude extract, it was anticipated that methodologycal innovations would be needed to separate all minor compounds correctly.

2. Experimental

2.1. Plant material

The aerial parts of *S. wolffii* were harvested in July 2001 from Herencsény, Hungary. A voucher specimen (collection number S94) has been deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

2.2. Reagents and standard ecdysteroid samples

Solvents of analytical grade were obtained from Reanal (Budapest, Hungary), and solvents of HPLC grade from Merck (Darmstadt, Germany). The reference ecdysteroids used as standards were available from earlier isolation work and were fully characterized in previous studies [**124-125**]. Their identities and purities were confirmed by NMR and NP- and RP-HPLC.

2.3. General experimental procedures

2.3.1. Procedures for separation

Classical CC was carried out on Brockman II. neutral alumina (Reanal, Budapest, Hungary) or Silica gel 60 (Merck, Darmstadt, Germany); **SPE** on Polyamid SC6 für die Säulenchromatographie (Woelm, Eschwege, Germany) (0.05-0.16 mm); and **gel-chromatography** on Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala, Sweden); **vacuum cyano-silica CC** (0.063-0.200 mm, Chemie Urticon-C-gel, Urticon, Switzerland) was used in one case, in NP mode with a gradient of *n*-hexane, *n*-hexane – acetone (9:1, 8:2, 7:3 and 6:4, v/v), and 50 ml fractions being collected (see *column 3*); **NP vacuum CC** on Kieselgel 60 GF-254 for TLC (Reanal, Budapest, Hungary); and **RP vacuum CC** on Kovasil C18 (0.06-0.02 μm) (Chemie Urticon-C-gel, Urticon, Switzerland) with a gradient of 30 to 60 % aqueous methanol, except in the case of *column 4* (from 50 to 85%). The proportion of

methanol was elevated by 5% after every five fractions. In each case, vacuum was achieved with a Sue 300E vacuum pump (Heto-Holten A/S, Gydevang, Denmark) and the flow rate was approximately 15 ml/min.

Analytical TLC was used for the monitoring of every chromatographic step up to HPLC. In **NP-TLC**, DC-Alufolien Kieselgel $60F_{254}$ (Merck, Darmstadt, Germany) was used with the following solvent systems: **1.** dichloromethane - ethanol (96%) (8:2, v/v); **2.** ethyl acetate - methanol - ammonia (25%) (85:15:5, v/v/v); **3.** toluene - acetone - ethanol (96%) - ammonia (25%) (100:140:32:9, v/v/v/v); **4.** dichloromethane - methanol - benzene (25:5:3, v/v/v); and **5.** ethyl acetate - ethanol (96%) - water (16:2:1, v/v/v). When **NP-TLC** was used on a **preparative** scale, the SP was DC-Platten 20 x 20 cm (Merck, Darmstadt, Germany).

In **RP-TLC**, Whatman $KC_{18}F$ sheets (Whatman, Clifton, NJ, USA) were used with the following solvent systems: **6.** methanol – water (4:6, v/v); **7.** acetonitrile – water (35:65, v/v); **8.** acetonitrile – trifluoracetic - acid (0.1%) (35:65, v/v); and **9.** tetrahydrofuran – water (45:55, v/v). In **CN-TLC**, HPTLC plates CN F_{254} 10x20 cm (5-7 μm) (Merck, Darmstadt, Germany) were used with the following solvent systems: **10.** *n*-hexane – acetone (6: 4, v/v); and **11.** acetonitrile – water (2:8, v/v). Below, solvent systems are denoted NP-TLC_1, NP-TLC_2...; RP-TLC_6, RP-TLC_7...; CN-TLC_{10} *etc.* Ecdysteroids were detected under UV light (Camag UV-lamp, Camag, Muttenz, Switzerland) at 254 nm (dark spots), after spraying with vanillin - sulfuric acid reagent [**126**] in UV light at 366 nm (fluorescent spots) and in daylight (usually olive-green spots for a short time).

NP-HPLC was used for the final purification of the ecdysteroids. Equipment used: a Jasco PU2080 HPLC pump and a Jasco UV2075 UV detector, connected to a Hercule 2000 chromatographic interface. Columns used: **1.** Zorbax SIL® (DuPont, Wilmington, DE, USA), 5 μm, 250 x 4.6 mm (analytical); **2.** Zorbax SIL® (DuPont, Wilmington, DE, USA), 5 μm, 250 x 9.4 mm (preparative); **3.** Agilent Zorbax SIL (Agilent Technologies Inc., Palo Alto, CA, USA); and **4.** Hypersil (BST Co, Budapest) 6 μm, 250 x 4 mm. Below, NP-HPLC columns are denoted C1-C4. Solvent systems: **1.** dichloromethane – isopropanol – water (125:50:5, v/v/v); **2.** dichloromethane – isopropanol – water (125:40:3, v/v/v); **3.** dichloromethane – isopropanol – water (125:30:2, v/v/v); **4.** dichloromethane – isopropanol – water (125:25:2, v/v/v); **5.** dichloromethane – isopropanol – water (125:15:1, v/v/v); and **6.** cyclohexane – isopropanol – water (100:40:3, v/v/v). Below, solvent systems are are denoted NP-$HPLC_1$, NP-$HPLC_2$, *etc.* The column used is denoted with an upper index: NP-$HPLC_1^{C1}$, NP-$HPLC_1^{C2}$ *etc.*

RP-HPLC was also used for some analyses and to extend the available analytical data on the ecdysteroids. Columns used: **5.** Spherisorb®-5ODS2 (Phase Sep.) 5 μm, 250 x 4.6 mm; **6.** Lichrospher® 100 RP-18 (Merck KGaA, Darmstadt, Germany), 5 μm, 250 x 4.6 mm; **7.** Agilent Zorbax SB-C18 (Agilent Technologies Inc., Palo Alto, CA, USA), 5 μm, 250 x 4.6 mm. RP-HPLC columns are denoted C5-C7. Mobile phases used: **1.** acetonitrile – water (23:77, v/v); **2.** acetonitrile – water (6: 4, v/v); **3.** acetonitrile – water (16.5:83.5, v/v); and **4.** acetonitrile – water – dichloromethane (80:30:3, v/v/v). Solvent systems are denoted RP-HPLC$_1$, RP-HPLC$_2$, *etc.*; columns are denoted with an upper index, *e.g.* RP-HPLC$_1^{C5}$, RP-HPLC$_1^{C6}$, *etc.*

The flow rate was usually 1 ml/min or 0.7 ml/min in the case of analytical HPLC (NP and RP), and 2-4 ml/min for preparative NP-HPLC.

2.3.2. Calculations on HPLC chromatograms

Jasco Borwin v1.50 chromatographic software was used for the recording of chromatograms, with a measurement frequency of 5 points/s. In some cases (see sections **3.3** and **3.5**), mathematical calculations were used on the curves to obtain more data, as follows: each set of absorbance/time coordinates of the chromatogram was transported to a ".txt"-file, and data-pairs of this file were translocated to Microsoft Excel. After this, superposition, the averages of parallel chromatograms or derivative curves could be calculated, and the outcome of calculations could be visualized as Excel point-diagrams.

2.3.3. Procedures for structure elucidation

1. Melting points were measured with a Boetius apparatus (Dresden, Germany).
2. Optical rotations were measured with a Perkin-Elmer 341 polarimeter.
3. The UV spectra were recorded in MeOH with a Shimadzu UV 2101 PC spectrophotometer. FT-IR spectra (KBr) were recorded using a Perkin-Elmer Paragon 1000 PC FT-IR spectrophotometer.
4. ^{1}H, ^{13}C and 2D (COSY, NOESY, HMBC and HMQC) NMR spectra were recorded in MeOH-*d*4 and in DMSO-*d*6, chloroform-*d*1 or pyridine-*d*5 in Shigemi sample tubes at room temperature, with a Bruker Avance DRX-500 spectrometer. In the 1D measurements (^{1}H, ^{13}C and DEPT-135), 64 K data points were used for the FID. The pulse programs of the 2D experiments (gs-COSY, gs-HMQC, HMQC-TOCSY {mixing time = 100 ms}, gs-HMBC, NOESY {mixing time = 350 ms} and ROESY {mixing time = 300 ms}) were taken from the Bruker software library. The pulse programs of the ^{1}H, ^{13}C, COSY, HMQC, HMBC, HMQC-

TOCSY and NOESY measurements were taken from the Bruker software library. Chemical shifts are given on the δ-scale, referenced to the solvents (δ_C = 39.51 and δ_H = 3.31 in MeOH-d_4, δ_C = 77.05 and δ_H = 7.27 in $CHCl_3$-d_1, δ_C = 39.51 and δ_H = 2.51 in DMSO-d_6).

5. HRESIMS and FABMS were recorded on a Finnigan MAT 95SQ hybrid tandem mass spectrometer (Finnigan MAT, Bremen, Germany), and ESIMS-MS on a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan Ltd., San Jose, CA, USA).

2.4. Extraction and isolation

2.4.1. Extraction and prepurification of the crude extract

The dried herb (2 kg) of *S. wolffii* was milled and percolated with methanol (20 l) at ambient temperature. The methanolic extract was evaporated to dryness (232.9 g) and dissolved in 1250 ml of methanol, and acetone (600 ml) was added to the solution. The resulting precipitate was removed by decantation, then rinsed three times, each with 150 ml of methanol – acetone (1:1, v/v). The supernatant and the methanol – acetone solutions were combined and evaporated to dryness. The residue (166.9 g) was redissolved in methanol (700 ml), and acetone (700 ml) was added to the solution. The precipitate was washed twice with 150 ml of methanol – acetone (1:1, v/v). The supernatant and the methanol – acetone solutions were combined and evaporated to dryness. The residue (124.5 g) was dissolved in 50% aqueous methanol (500 ml) and extracted four times with *n*-hexane (4 x 1000 ml).

The aqueous methanolic phase was evaporated to dryness, and the residue (108 g) was dissolved in methanol (100 ml) and adsorbed on silica gel (200 g), using a rotatory evaporator. This was added to the top of a previously packed column of silica (1000 g, *column 1*; 1010 x 55 mm*) suspended in dichloromethane. After the column had been extensively washed and conditioned with dichloromethane (4.8 l), the ecdysteroids were eluted with dichloromethane – ethanol (96%) (95:5, 9:1, 85:15, 8:2 and 7:3 v/v) (8, 8.8, 7.2, 7.2 and 4 l, respectively) and 800 ml fractions were collected. The progress of the elution was monitored by the use of NP-TLC, using solvent systems 3, 4 and 5.

2.4.2. Isolation of ecdysteroids

Fractions 11-13 of *column 1*, eluted with dichloromethane –ethanol (96%) (95:5, v/v) were combined and evaporated to dryness. The dried residue (14.3 g) was dissolved in 20 ml of methanol. The solution was mixed with 30 g of polyamide and taken to dryness by rotatory

* Column sizes are indicated as length x diameter.

evaporation. The sample was applied onto the top of a column of 30 g of polyamide (*column 2*; 300 x 25 mm). Five fractions (100 ml each) were collected, in elutions with plain water, and water – methanol (9:1, 8:2, 7:3 and 1:1, v/v) respectively. The second fraction, eluted with water – methanol (9:1, v/v), was evaporated to dryness, and the residue (2.16 g) was dissolved in 10 ml of methanol, adsorbed onto 3 g of CN as described previously, and applied onto a column of 18 g of CN (*column 3*; 180 x 20 mm). Gradient elution was performed as described in section **2.3.1.** Fractions 39-66, eluted with *n*-hexane – acetone (9:1, v/v) were combined and evaporated to dryness. The residue (0.17 g) was dissolved in 1.5 ml of methanol, 0.5 ml of water was added to the solution, and it was applied onto a column of octadecylsilica (*column 4*; 300 x 20 mm). Gradient elution was performed with water – methanol (see section **2.3.1**), and 20 ml fractions were collected. Fractions 28-30, eluted with water – methanol (25:75, v/v) were combined and evaporated, and the dry residue (4.5 mg) was separated by using NP-HPLC$_5$, to obtain compounds **1** (1.5 mg) and **2** (2.9 mg). Fractions 22 and 23 eluted with water – methanol (3:7, v/v), were also combined and purified by NP-HPLC$_3$ to yield compound **3** (3.5 mg).

Fractions 27-30 from *column 1*, eluted with dichloromethane – ethanol (96%) (9:1 and 85:15, v/v), were combined and evaporated to dryness. The dried residue (4.3 g) was dissolved in 5 ml of methanol. The solution was mixed with 9 g of polyamide and taken to dryness by rotatory evaporation. The sample was adsorbed onto polyamide and packed in a G3 glass filter (Pyrex, France) (*column 5*; 90 x 25 mm). Elution was carried out with water, 25% and 50% aqueous methanol and pure methanol (100 ml of each), and 100 ml fractions were collected. The fractions eluted with water and with 25% aqueous methanol were combined and evaporated to dryness. The dry residue (3.5 g) was dissolved in methanol (5 ml) and adsorbed on silica (10 g), which was then added onto the top of a previously packed column of silica (35 g), suspended in ethyl acetate – methanol – water (85:10:5, v/v/v; *column 6*; 65 x 30 mm). Vacuum CC was used with isocratic elution (ethyl acetate – methanol – water, 85:10:5, v/v/v) and 14 fractions of 25 ml were collected. Fractions 3-6 were subjected to repeated crystallization in ethyl acetate – methanol (2:1, v/v) to yield compound **4** (427 mg).

The mother liquid and the dry residue of fractions 1, 2 and 7-11 (2.25 g) were combined and separated by vacuum CC on silica (40 g) (*column 7*; 65 x 31 mm). The components were eluted with a stepwise gradient of dichloromethane, dichloromethane – ethanol (98:2, 95:5 and 9:1, v/v) and methanol (250, 75, 175, 1025 and 75 ml, respectively) and 25 ml fractions were collected. Fractions 22-25 (0.43 g) eluted with dichloromethane – ethanol (9:1, v/v) were combined, and fractionated by gel chromatography on Sephadex LH$_{20}$ (24 g), using

ethyl acetate – methanol – water (16:2:1, v/v/v) as eluent (*column 8*; 560 x 20 mm). 2 ml fractions were collected. Fractions 36-48 (0.24 g) were combined and separated by preparative NP-TLC_2. The final purification of the ecdysteroid obtained by TLC was carried out by NP-$HPLC_3$, to result in compound **5** (5 mg).

Fractions 35-39 from *column 7* (0.38 g) were also combined and fractionated on Sephadex LH_{20} (*column 9*; 560 x 20 mm) in the same way as in the case of fractions 22-25 from *column 8*. Fractions 32-40 from *column 9* (0.158 g) were further separated by Sephadex gel chromatography (13 g) (*column 10*; 570 x 10 mm), using ethyl acetate – methanol (2:1, v/v) as eluent, and 1 ml fractions were collected. Fractions 11-16 (0.037 g) were finally purified by using preparative NP-TLC_2 and NP-$HPLC_3$ to yield compound **6** (7.2 mg). Fractions 51-60 (0.18 g) from *column 9* were fractionated by preparative NP-TLC_2 and NP-$HPLC_2$ to obtain compound **7** (18 mg). Fractions 40-46 (0.51 g) from *column 7* were purified by repeated use of Sephadex gel chromatography (*column 11*; 560 x 20 mm, and *column 12*; 570 x 10 mm) in the same way as in the case of fractions 35-39 from *column 7*. First, ethyl acetate – ethanol – water (16:2:1, v/v/v) was used and 2 ml fractions were collected. Fractions 22-25 (0.08 g) from *column 11* were further separated by Sephadex gel chromatography (*column 12*) again and the ecdysteroids were eluted with ethyl acetate – methanol (2:1, v/v); 2 ml fractions were collected. Fractions 6-10 (0.04 g) were purified by using NP-$HPLC_3$ to yield compound **8** (13 mg).

Fractions 29-38 from *column 11* (0.179 g) were recycled onto the same Sephadex column (*column 11'*) and the ecdysteroids were eluted with ethyl acetate – ethanol – water (16:2:1, v/v/v); 2 ml fractions were collected. Fractions 45-63 (0.08 g) from this column were purified by NP-TLC_2 and NP-$HPLC_3$ to yield compound **9** (24 mg).

Fractions 47-63 from *column 7* were combined and evaporated to dryness. The dry residue (0.63 g) was dissolved in methanol and adsorbed onto 2 g of alumina. This was added to the top of a previously packed column of alumina (18 g), suspended in chloroform (*column 13*; 170 x 15 mm). The ecdysteroids were eluted from the alumina with a stepwise gradient of chloroform – methanol (95:5, 9:1, 85:15 and 8:2, v/v; 370, 1160, 620 and 200 ml, respectively) and 10 ml fractions were collected. Fractions 44-138 (0.26 g), eluted with chloroform – methanol (9:1, v/v), were combined and subjected to vacuum CC on 180 g of octadecylsilica (*column 14*; 450 x 30 mm). Elution was carried out as described in section **2.3.1**, and 50 ml fractions were collected. Fractions 11-13, eluted with 40% aqueous methanol, gave compound **10** (2.2 mg). Fractions 23-28 (0.13 g), eluted with 50% and 55% aqueous methanol, were further purified by NP-$HPLC_2$ to obtain compound **11** (28 mg).

Fractions 31-35 from *column 1*, eluted with dichloromethane – ethanol (96%) (85:15, v/v), were combined. The dry residue (13,6 g) was dissolved in 10 ml of methanol and adsorbed onto 68 g of alumina, which was added to the top of 400 g of alumina previously packed into a column (*column 15*; 400 x 45 mm). Stepwise gradient elution was carried out with 9:1, 85:15, 8:2 and 7:3 v/v mixtures of dichloromethane – ethanol (11.1, 4.1, 1.7 and 1.8 L, respectively) and 100 ml fractions were collected. Fractions 24-30 (0.67 g), eluted with dichloromethane –ethanol (9:1, v/v), were separated by using preparative NP-TLC_5, which gave three well-defined zones. The ecdysteroids of these zones were further purified by NP-$HPLC_4$ to furnish compounds **12** (3 mg) and **13** (0.7 mg) from the first, compounds **14** (1.7 mg) and **15** (2.7 mg) from the second, and compound **16** (0.7 mg) from the third zone.

Fractions 31-90 from *column 15* were subjected to repeated crystallization from ethyl acetate – methanol (2:1, v/v) to yield compound **17** (1.22 g). Fractions 91-190 from *column 15* (0.67 g) were separated, using RP vacuum CC on 180 g of octadecylsilica (*column 16*; 450 x 30 mm) as described above, and 50 ml fractions were collected. Fractions 15-17 (3 mg), eluted with 45% aqueous methanol, were separated by NP-$HPLC_2$ to give compounds **18** (0.7 mg) and **19** (0.7 mg). Fractions 21-23 (85 mg), eluted with 50% aqueous methanol, were also separated by NP-$HPLC_2$, and 5 compounds were isolated: compounds **11** (0.6 mg) and **17** (11.7 mg) again, and **20** (8.4 mg), **21** (1.8 mg) and **22** (9.8 mg). Fractions 28-32, eluted with 55 and 60% aqueous methanol (15.2 mg dry residue), gave compound **12** (6.8 mg) after purification by NP-$HPLC_2$.

Fractions 41-46 (9.1 g) of *column 1*, eluted with dichloromethane - ethanol (96%) (7:3, v/v), were adsorbed on 45 g of alumina and the sample adsorbed on the alumina was packed onto the top of a column of 270 g of alumina (*column 17*; 255 x 35 mm). Gradient elution was carried out with chloroform – ethanol (96%) (9:1, 8:2, 7:3, 65:35, 6:4, v/v) (1700, 1800, 4900, 8900 and 2900 ml respectively), and 100 ml fractions were collected. Fractions 42-60 (0.36 g), eluted with chloroform – ethanol (96%) (7:3, v/v) were combined and further fractionated, using preparative NP-TLC_5. The final purification was made by NP-$HPLC_3$ to yield compound **23** (9.9 mg). Fractions 91-96 (0.11 g), eluted with chlorophorm-ethanol (65:35, v/v) were also purified by NP-TLC_5, and NP-$HPLC_1$, and compound **24** (10 mg) was isolated. Fractions 97-108 (0.23 g) from *column 17*, eluted with chloroform – ethanol (96%) (65:35, v/v), were also purified by NP-TLC_5 and NP-$HPLC_1$ to give compound **25** (11 mg). The scheme of the isolation is outlined in **Fig. 3**.

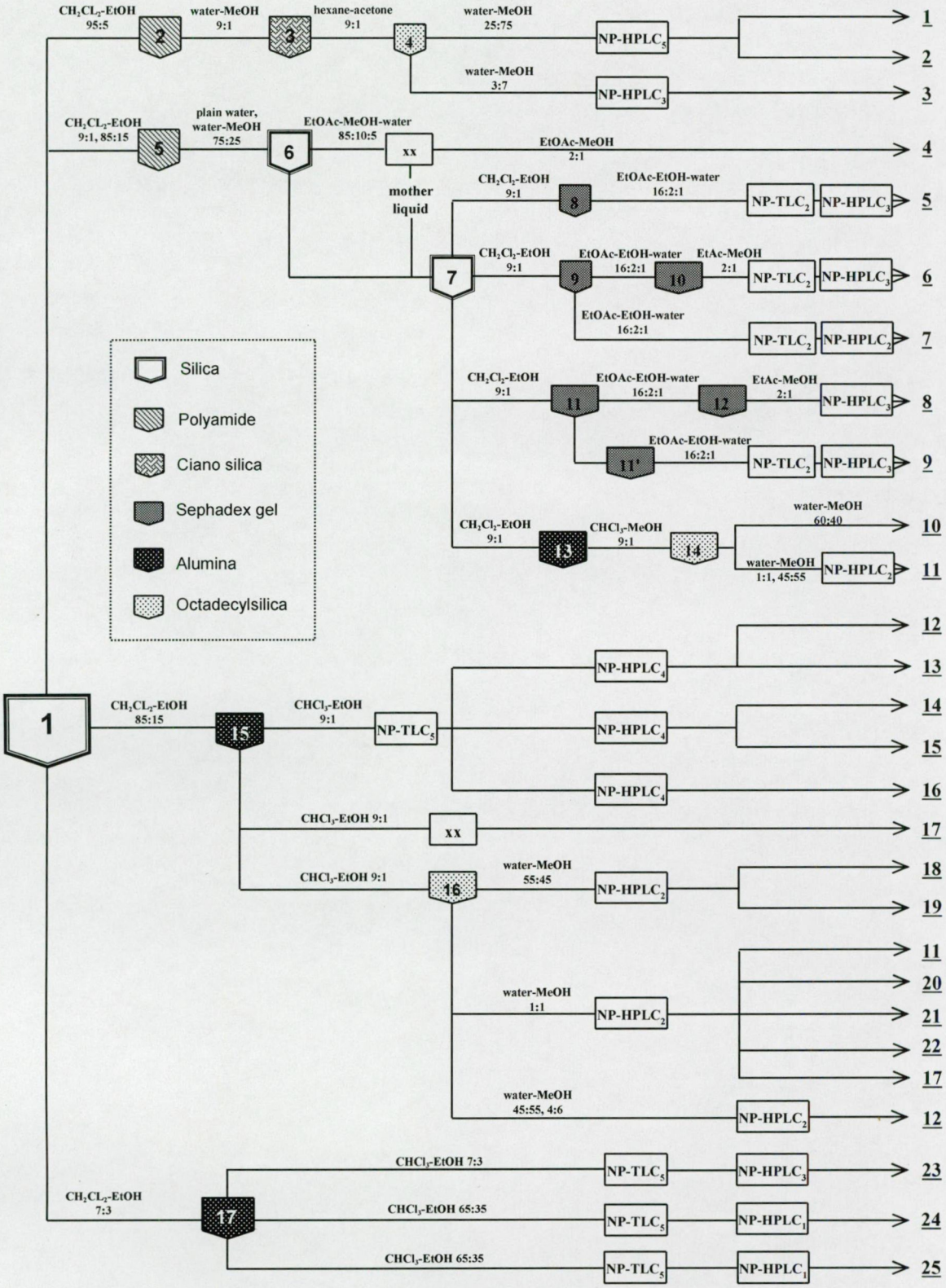


Figure 3. Chromatographic procedure of ecdysteroid isolation.

Numbers of compounds: **1** ajugasterone C 2,3;20,22-acetonide, **2** 20E 2,3;20,22-acetonide, **3** ajugasterone C 20,22-acetonide, **4** polypodine B, **5** ajugasterone D, **6** dacryhainansterone, **7** pterosterone, **8** 20E 20,22-acetonide, **9** makisterone C, **10** 11α-hydroxypoststerone, **11** ajugasterone C, **12** 22-deoxy-20E, **13** 5α-20E, **14** E, **15** makisterone A, **16** 22-deoxy-20,21E, **17** 20E, **18** herkesterone, **19** 25-hydroxydacryhainansterone, **20** isovitexirone, **21** 14-epi-20E, **22** 3-epi-20E, **23** 22-epi-20E, **24** turkesterone, **25** 20,26E

2.5. Application of peak cutting

Peak cutting is a new preparative HPLC method developed in our laboratory; it is described in section **3.3**. The method was always carried out as follows:

- The elution time of the solvent used for the peak cutting was determined by a number of injections.
- The chromatographic software was set to automatic start.
- The sample to be separated was loaded into the loop of 100 µl, the injector was turned into the "inject" stage (the recording of the chromatogram was started) and at the same time the stopwatch was set.
- As time of the difference between the intersection time of the overlapping ecdysteroid peaks and elution time of cutting solvent was came, 100 µl of it was immediately injected.

2.6. Elimination of the 11α-OH group

An olefin bond was formed between C-9 and C-11 in the case of 4 ecdysteroids containing an 11α-OH group as follows:

1.0 mg of turkesterone, isovitexirone, 11α-hydroxypoststerone or ajugasterone C 2,3;20,22-diacetonide was dissolved in 2 ml of methanol. Each solution was adsorbed in a rotatory evaporator onto 0.2 g of Brockman II neutral alumina chromatographic SP (Reanal, Budapest, Hungary), which had been previously washed with 2 ml of methanol. The temperature was 50 °C. After the adsorption, the products were immediately eluted from the adsorbent with 1.4 ml of methanol using a G4 filter. NP-HPLC was used for the monitoring of the products at 298 and 242 nm.

2.7. Preparation of extract to establish the presence of dienones in the plant

To examine the presence of 7,9(11)-diene ecdysteroids in the plant, a prepurified extract was used, which was prepared as follows:

118 g of the leaves of *S. wolffii* was extracted with 5 times 1000 ml of methanol, using an ultrasonic bath for 15 min in each case. The methanolic extract was evaporated, and the residue (23.5 g) was prepurified by precipitation with increasing amounts of acetone as described in section **2.4.1**, acetone being added to the methanolic solutions in amounts to achieve methanol:acetone ratios of 2:1, 1:1 and 1:2, v/v. Each time, the precipitate was removed and washed 3 times with the solvent it was precipitated from. The remaining solutions were combined and evaporated, and the residue (14.2 g) was used for the investigations described in section **3.5**.

3. Results

3.1. Clean-up and chromatographic separation

The methanolic extract of *S. wolffii* was found to contain a wide spectrum of various compounds. Fractionated precipitation with increasing amounts of acetone, and solvent-solvent participation between *n*-hexane and water – methanol (1:1, v/v) allowed purification of the ecdysteroids from both the polar and apolar contaminants. After these steps, the extract was further purified by using large-scale classical adsorption chromatography on silica. A high ratio of analyte/SP (1:10) was used and the ecdysteroids were enriched with a preconditional wash with dichloromethane. With a six-steps gradient (dichloromethane – methanol; 95:5, 9:1, 85:15, 8:2 and 7:3, v/v), the ecdysteroids could be selectively eluted from the SP, and the matrix components were not eluted even at a dichloromethane – methanol ratio of 8:2, v/v. On the basis of the polarity, four groups of fractions containing ecdysteroids could be separated. The two least polar fractions (fractions 11-13 and 27-30 from *column 1*) contained large amounts of flavonoid aglycones. These constituents could be separated well from the ecdysteroids, using further purification on polyamide [**127**].

After the preliminary purification, NP chromatographic methods were used on CN, silica and alumina. At this level of the separation, high amounts of 20E (**17**) and polypodine B (**4**) could be isolated by repeated crystallization using ethyl acetate – methanol (2:1, v/v). After NP separation, vacuum RP-CC was used in the case of the relatively apolar ecdysteroids and those of medium polarity. With this method, a new natural compound, 11α-hydroxypoststerone (**10**) could be isolated without further purification [**128**]. **Fig. 4** shows the RP-HPLC chromatogram of the prepurified extract (before its application onto *column 1*) and that of the isolated pure compound.

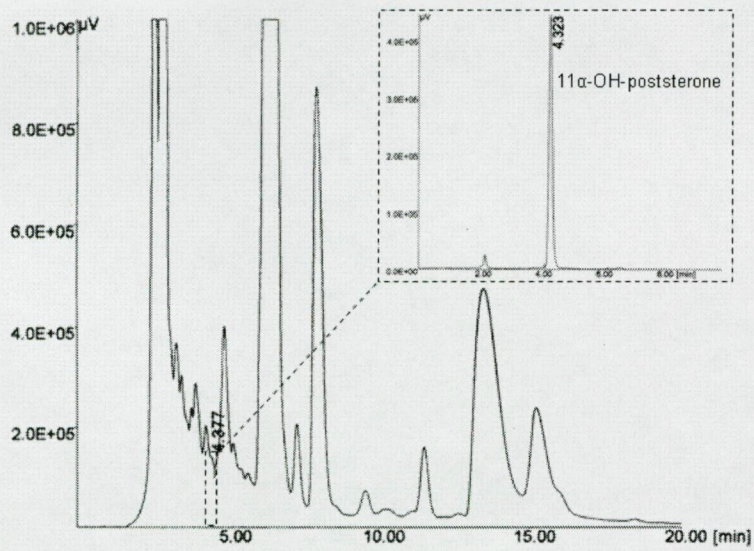


Figure 4. RP-HPLC chromatogram of the extract and the isolated 11α-hydroxypoststerone

Water - methanol (1:1, v/v) eluent was used on column C7 at a flow rate of 0.9 ml/min, at wavelength of 254 nm.

Sephadex gel filtration was also used with multiple recyclization at this level of separation. The next step in the isolation was preparative TLC on silica plates; and through the use of NP-HPLC, pure compounds could be obtained.

3.2. HPLC methods in the final purification

In the separation of multicomponent samples, it was often necessary to use special HPLC methods. Recyclization techniques were of great assistance, but heart-cut, virtual separation with dual UV detection, serial column junction and peak cutting (see section **3.3**) were also used.

To take better advantage of the whole length of the HPLC columns during preperative work, sample injections followed each other as soon as possible without heavy contamination of the compound to be isolated. **Fig. 5** illustrates such a separation. The isolated compound was 90% pure, and could be simply purified further by recyclization. After structure elucidation, it was proven to be ajugasterone C 20,22-acetonide (**3**), a new compound from *S. wolffii*.

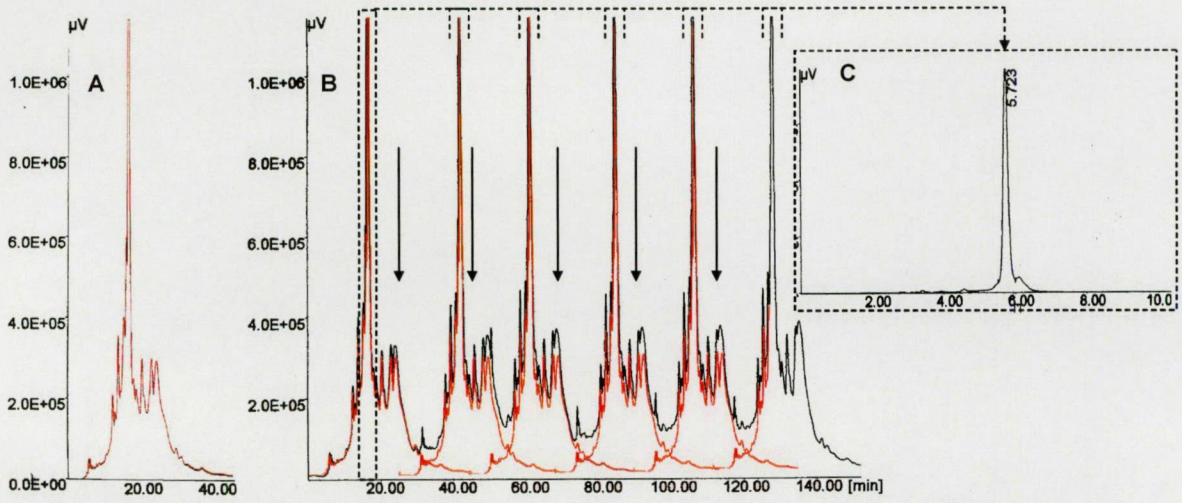


Figure 5. Isolation of ajugasterone C 20,22-acetonide (**3**) by fast repeated injections

The HPLC chromatogram of fraction 22 and 23 of *column 4* can be seen in **fig. 5A**. This fraction was purified by fast repeated injections, as shown in **Fig. 5B**. Arrows indicate injection times, while red curves denote copies of the chromatogram of the single injection (pasted by computer using Adobe Photoshop 7.0CE). The column was overloaded (1.684 mg in 100 µl of sample; 3 ml/min, NP-HPLC$_3^{C2}$). **Fig. 5C** shows the analytical chromatogram of the isolated peak (NP-HPLC$_3^{C1}$, 1 ml/min).

Besides the ecdysteroids with the usual 7-en-6-one structure, *S. wolffii* also contains ecdysteroids with a 7,9(11)-dien-6-one structure. Because of the extented conjugation, the UV absorbance maximum of these molecules shifts from the usual 240-260 nm to higher

wavelength of approximately 300 nm. Since the 7-en-6-one ecdysteroids have only infinitesimal molar absorbance at such high wavelength, the ecdysteroids with dienone structure could be selectively detected. Detection at these two wavelengths facilitates sample selection and fraction collection. **Fig. 6** shows the chromatograms of the fraction containing herkesterone (**18**) and 25-hydroxydacryhainansterone (**19**), two new natural compounds.

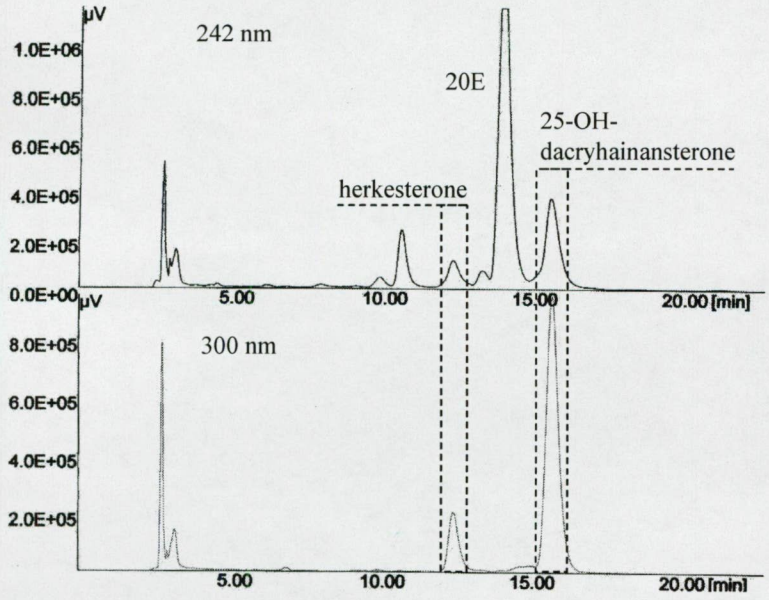


Figure 6. Dual UV detection in screening of dienones

Ecdysteroids with a 7,9(11)-diene-6-one structure show higher UV-absorption at the wavelength of 300 nm, while the peaks of regular 7-en-6-one ecdysteroids (like 20E) show a great decrease in area.

Detection at these two parallel wavelengths was essential in the isolation of dacryhainansterone (**6**), and also to monitor the 11α-OH elimination (see sections **2.6** and **3.4**), and to establish the presence of ecdysteroids with a dienone structure in the extract (see section **3.5**).

3.3. The concept and development of peak cutting, a new HPLC method

A special method of preparative HPLC was developed to increase the separation of two overlapping peaks. The component with the lowest elution force in the solvent system used was injected during the development in such a way that the eluting solvent peak appeared exactly between the two overlapping peaks (see also in section **2.5**). This resulted in an increased retardation of the later-eluting peak, and promoted a more effective separation. **Fig. 7A** shows the chromatogram of the co-eluting isovitexirone (**20**) and an unknown ecdysteroid. Since pure dichloromethane has a different refraction coefficient from that of the solvent system, depending on the wavelength it can be detected (denoted **D**). The time of elution of the dichloromethane was determined with several injections. The chromatogram of the isolated 14-epi-20E (**21**), which is a new natural compound, can be seen in **Fig. 7B**.

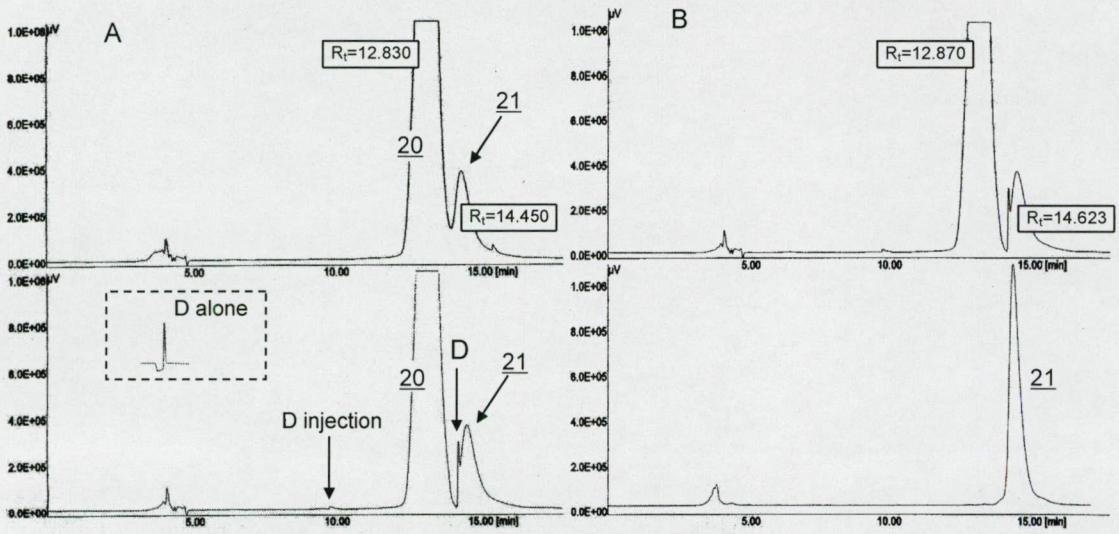


Figure 7. Peak cutting separation on NP-HPLC with dichloromethane

Fig. 7A illustrates the process of peak-cutting, and **Fig. 7B** demonstates the achievement. The solvent system was dichloromethane - isopropanol - water (125:40:3, v/v/v) at a flow rate of 0.7 ml/min, with detection at 254 nm. 100 µl of dichloromethane was injected at 9.81 min. Affected retention times (R_t) are also presented.

Peak cutting was also used for the isolation of 3-epi-20E (**22**). The eluent was cyclohexane – isopropanol – water (100:40:3, v/v/v) and cutting was performed with cyclohexane.

The method was applied with RP-HPLC too. 100 µl of water was used to cut the overlapping peaks of a fraction containing two compounds, 20E (**17**) and 3-epi-20E (**22**). Methanol – water (6:4, v/v) was used as eluent on column C7, and the separation was followed by detection at 245 nm. Elution of injected water causes only a slight uncertainty in the baseline at this wavelength, but its detection is possible at 200 nm, which is essential for calculation of an adequate injection time. However, water undergoes retention on the stationary phase, and its elution lasts for approximately 1 min. Serial measurement was performed to decide which part of the solvent peak plays an effective role in peak cutting. **Fig. 8** shows the results of the chromatographic analysis.

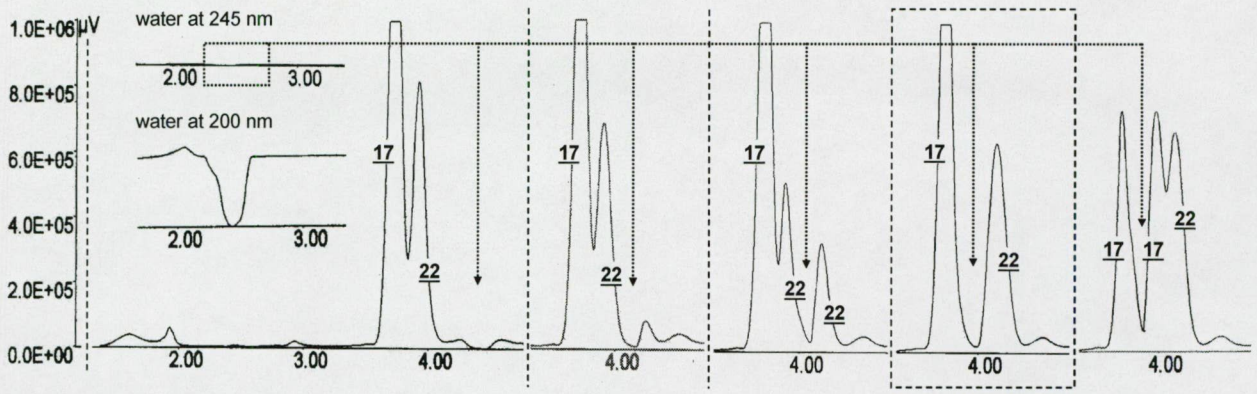


Figure 8. Peak cutting with various timings of water injections

Five chromatograms of the peak-cut separation of 20E (**17**) and 3-epi-20E (**22**) are presented. The solvent system was methanol - water (6:4, v/v), at an eluent flow of 1 ml/min at 245 nm. In each case, 100 µl of water was injected at different times (1.96, 1.65, 1.55, 1.30 and 1.25 min, respectively). Chromatograms of clean water detected at 245 nm and 200 nm are also depicted. Arrows show the effect of water.

As **Fig. 8** reveals, the strongly decreasing solvent force can not only cut the chromatogram between the peaks of two different compounds, but is also able to duplicate a peak of one pure compound. Although the use of an accurately timed flush of the cutting solvent seemed to lead to ideal separation (dash-lined frame in **Fig. 8**), some quantities of both compounds may have remained overlapped. To examine this phenomenon, the separation was simulated by developing the pure compounds separately. Five parallel developments were made with 20E and 3-epi-20E separately, and then five separate developments with both compounds, using the water injection of 100 µl at 1.30 min were also performed. 5 µl of 20E (1.89 mg/ml; 9.45µg) and 10 µl of 3-epi-20E (0.96 mg/ml; 9.6 µg) were injected. Two coinjected developments were also performed with these quantities of both compounds and with the injection 100 µl of water at 1.30 min. One of these developments was performed before the four series of simulation, and the other one was performed after it.

Each of the four series of separately developed chromatograms was averaged as described in section **2.3.2**. **Fig. 9A** shows these two theoretical chromatogram pairs; magnified overlapping areas are presented in the box denoted by dashed lines. The superposition calculated by mathematical summation of the data is indicated with a red line, which corresponds to the two chromatograms of the coinjected and cut compounds, as shown in **Fig. 9B**.

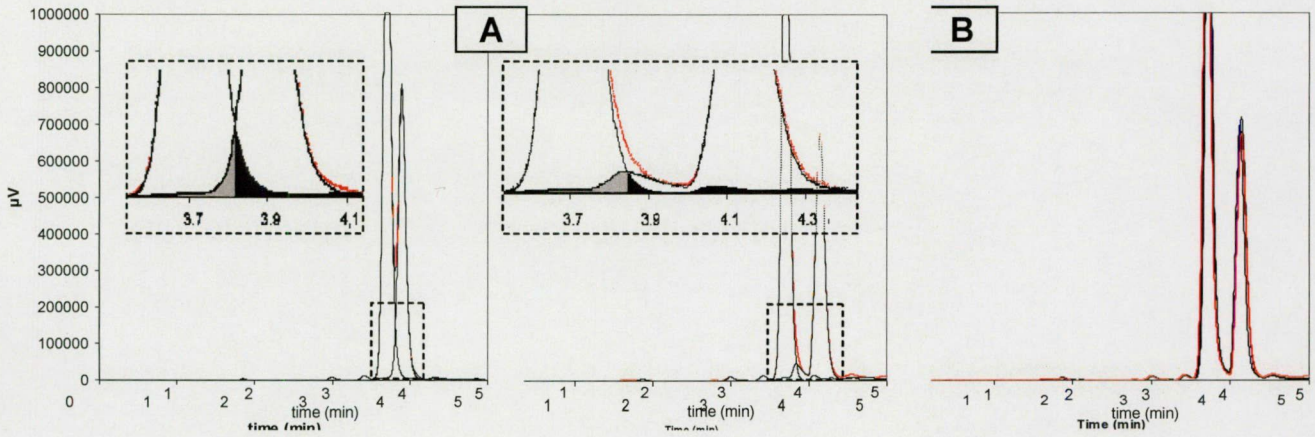


Figure 9. Demonstration of peak cutting with averaged chromatograms.

In the magnified chromatogram parts on **Fig. 9A** (dashed boxes), black zones indicate the amount of compound **17** remaining overlapped with the peak of compound **22**, and grey zones show the reverse case (with the appropriate collection of fractions). **Fig. 9B** depicts the correspondence between the calculated superposition and the chromatograms of the coinjected and cut compounds (two black curves represented). Averaged superpositions of the corresponding chromatograms are to be seen in red.

The Figure also shows that the ideal time for a change in fraction collecting is somewhat earlier than the minimum of the superpositioned curve.

Each series was aligned according to the retention times, and paired. **Table 2** shows the retention times (R_t) and the calculated selectivity factors (α) for the selected pairs.

Table 2. Comparison of retention times and selectivity factors with and without peak cutting.

	Common procedure			Peak cutting		
	R_t ^{20E} (min)	R_t $^{3\text{-epi }20E}$ (min)	α	R_t ^{20E} (min)	R_t $^{3\text{-epi }20E}$ (min)	α
1	3.660	3.890	1.063	3.653	4.143	1.134
2	3.660	3.893	1.064	3.660	4.147	1.133
3	3.663	3.893	1.063	3.663	4.147	1.132
4	3.670	3.897	1.062	3.663	4.150	1.133
5	3.677	3.900	1.061	3.663	4.167	1.138
Mean	3.666	3.895	1.062	3.660	4.151	1.134
(+/- SD)	(+/- 0.00738)	(+/- 0.00391)	(+/- 0.00117)	(+/- 0.00434)	(+/- 0.00939)	(+/- 0.00214)

The overlapping areas of each chromatogram pair obtained by this method were calculated in the following way:

- The time of intersection of the two peaks was determined.
- Both peaks were integrated in two parts, from the beginning of the peak to the intersection with the other peak, and from the intersection to the end of the peak.

- The theoretical change in the collection of fractions was presumed to be the intersection time; thus, the tailing part of the earlier eluting peak and the leading part of the later one were taken as loss on the one hand and as contamination on the other hand (see also grey and black areas in **Fig. 9A**)

Since the overlapping areas were almost 2 orders of magnitude less than the amounts injected, calibrating lines were determined for both compounds and were taken as the basis for calculation of quantitative data (**Fig. 10**).

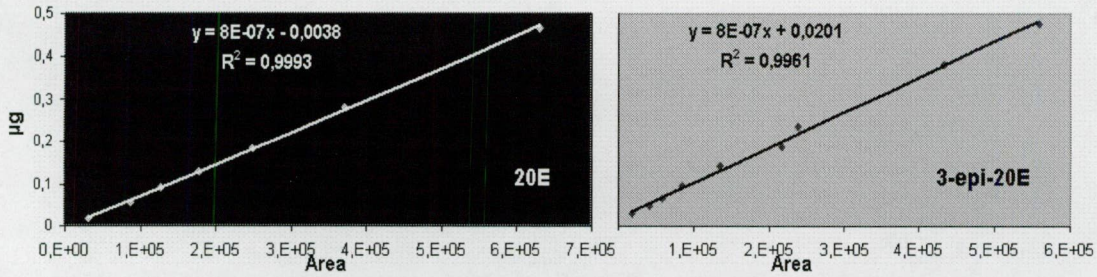
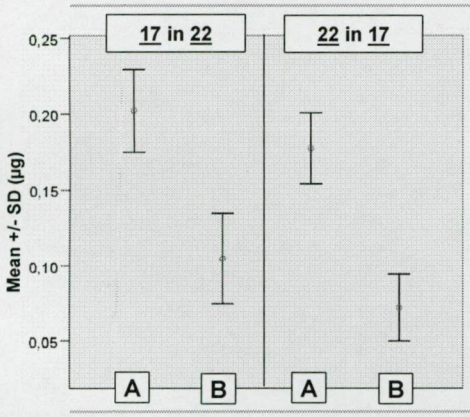


Figure 10. Calibration to quantify the overlapping amounts of 3-epi-20E and 20E.

Seven developments were performed in the case of 20E (injecting 0.0187; 0.0561; 0.0935; 0.1309; 0.187; 0.2805 and 0.4675 µg, respectively) and nine developments in the case of 3-epi-20E (injecting 0.0561; 0.0935; 0.1309; 0.187; 0.2805; 0.374; 0.4675; 0.748 and 0.935 µg, respectively). The equations of the lines were taken as the basis of the calculation of the overlapping quantities from the integrated areas for both compounds.

The differences between the usual chromatographic technique and the peak cutting procedure as concerns the overlapping of the two compounds were measured and confirmed with one-sample T-tests at a significance level of 95%, using SPSS for Windows 14.0. For both compounds, a significant decrease of contamination could be achieved. **Fig. 11** illustrates the results of the analysis.



20E (17) in 3-epi 20E (22)	Mean (µg)	Std. Deviation	Std.Error of mean	t	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the difference Lower	Upper
A	0.2021	0.02712	0.01213	16.666	0.000	0.20214	0.1685	0.2358
B	0.1045	0.03005	0.01344	7.779	0.001	0.10455	0.0672	0.1419

3-epi 20E (22) in 20E (17)	Mean (µg)	Std. Deviation	Std.Error of mean	t	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the difference Lower	Upper
A	0.1773	0.02331	0.01043	17.010	0.000	0.17733	0.1484	0.2063
B	0.0720	0.02236	0.01000	7.200	0.002	0.07199	0.0442	0.0998

Figure 11. Differences in overlapping between developments with and without peak cutting

The left side of the error bar shows the amounts of 20E remaining overlapped with 3-epi-20E in µg, and the right side shows the reverse case. On both sides, **A** denotes the conventional separation and **B** the case when peak cutting was used. 5 parallel measurements were performed; the Table presents data from the statistical analysis.

Accessible yield and purity (if they would be coinjected and separated at the intersection time) of both compounds was calculated using the injectioned amounts and the overlapped quantities obtained from the calibration, results are shown in **Table 3**.

Table 3. Improvement in separation of 20E and 3-epi-20E if peak cutting is used

The Table shows the attenuable yield and purity of both compounds. Amounts of contaminants are given as mass percentages of the theoretically isolated peak (if the compounds were coinjected) according to the formula:

$$\text{contamination\%}_{20E} = [\,1 - (m_{\text{inj.20E}} - m_{\text{lost 20E}}) / (m_{\text{inj.20E}} - m_{\text{lost 20E}} + m_{\text{lost 3-epi-20E}})] \times 100$$

	20E				**3-epi 20E**			
	Yield (%)		Contamination (m/m %)		Yield (%)		Contamination (m/m %)	
	Conventional	Peak-cut	Conventional	Peak-cut	Conventional	Peak-cut	Conventional	Peak-cut
1	98.06	98.90	1.94	1.10	95.26	97.39	4.74	2.61
2	98.29	99.14	1.71	0.86	95.73	97.05	4.29	2.95
3	97.92	99.50	2.08	0.50	95.75	97.63	4.25	2.37
4	98.45	99.41	1.55	0.59	96.55	98.67	3.44	1.33
5	97.87	99.22	2.10	0.78	95.09	98.11	4.91	1.89
Mean	**98.12**	**99.24**	**1.88**	**0.76**	**95.68**	**97.77**	**4.32**	**2.23**
(+/- SD)	**(+/- 0.25)**	**(+/-0.24)**	**(+/- 0.25)**	**(+/- 0.24)**	**(+/- 0.58)**	**(+/- 0.63)**	**(+/- 0.58)**	**(+/- 0.63)**

3.4. Preparation of ecdysteroid 7,9(11)-dien-6-ones

Four 11α-hydroxy-ecdysteroids (ajugasterone C 2,3;20,22-diacetonide, turkesterone, isovitexirone and 11α-hydroxypoststerone) isolated from *S. wolffii* were dehydrated to obtain dienone structures. The transformation took place as described in section **3.5**, and HPLC was used to determine yields. **Fig. 12** shows the NP-HPLC chromatogram of isovitexirone (above) and the 24,25-didehydrodacryhainansterone obtained (below).

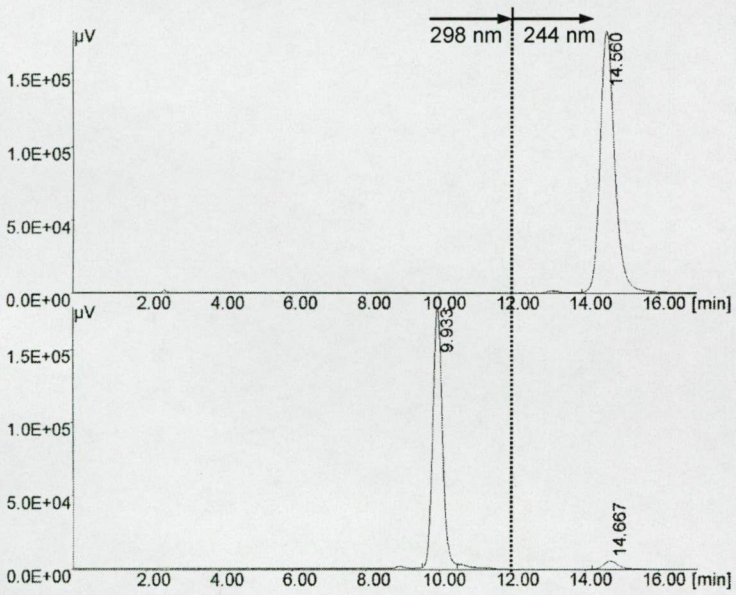


Figure 12. Analysis of water elimination from isovitexirone

The NP-HPLC$_6$ system was used at a flow rate of 1 ml/min. Wavelength detection was changed at 12 min in both cases

To calculate the molar ratio (and yield), the chromatograms of the products were used. The ratio of peak areas (product/original compound) was calculated, and corrected for the molar absorption ratio of the compounds ($\varepsilon_{original}^{244nm}/\varepsilon_{product}^{298nm}$).

The compounds obtained and their yields were: 25-hydroxydacryhainansterone (85.66%, *n/n*) from turkesterone (**24**), 25,26-didehydrodacryhainansterone (93.76%, *n/n*) from isovitexirone (**20**) (shown above), 9,11-didehydropoststerone (93.92 %, *n/n*) from 11α-hydroxypoststerone (**10**), and dacryhainansterone 2,3;20,22-diacetonide (96.56 %, *n/n*) from ajugasterone C 2,3;20,22-diacetonide (**1**). Each product was identified by ^{1}H and ^{13}C NMR spectroscopy too; the latter three compounds are new ecdysteroids.

3.5. Verification of the dienone presence in the plant

Since the chromatographic SP catalysed the dehydration described above, it might be suggested that the isolated dienones are artefacts of 11α-hydroxyecdysteroids, formed during the isolation procedure. To examine this, the extract of *S. wolffii* was analysed by RP-HPLC and 2D NP-TLC. A prepurified extract (using only fractionated precipitation with acetone, as described in section **2.7.**) was used. **Fig. 13** shows the chromatograms of the extract detecting on both 235 and 298 nm (the two UV absorption maxima of dacryhainansterone) according to the dual detection described previously (see page 20). **Fig. 14** presents the outcome of the TLC measurement.

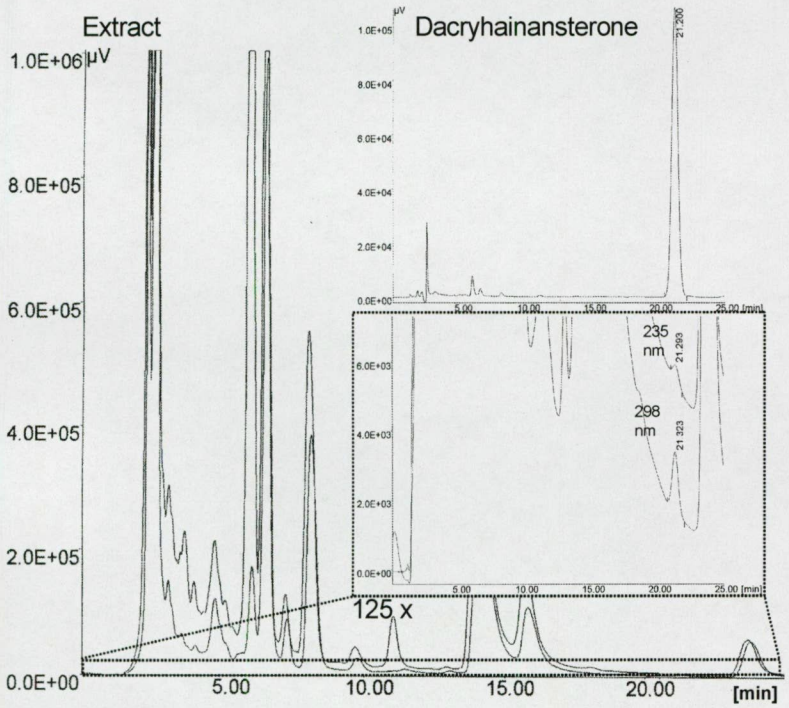
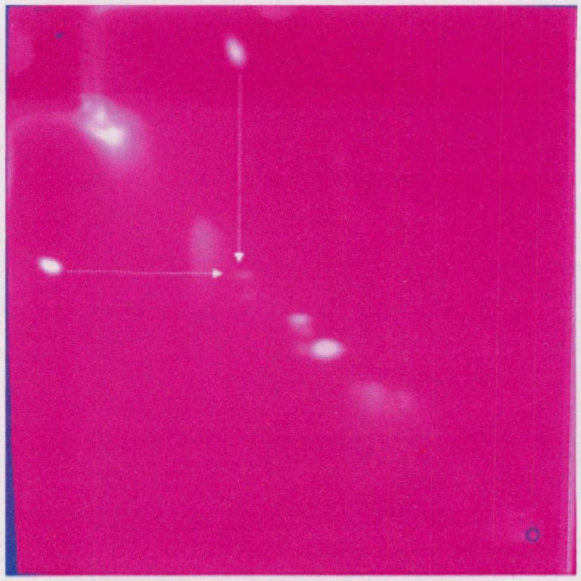


Figure 13. Dual UV detection of dacryhainansterone in the extract
Water - methanol (1:1, v/v) was used as eluent at a flow rate of 1 ml/min on column C7. The 125x zoom shows that the peak corresponding with that of dacryhainansterone sharpens at 298 nm as compared with 235 nm. The chromatogram of pure dacryhainansterone is shown above the magnified chromatogram.

Figure 14. 2D NP-TLC of the extract.

Multiple development was used in one dimension, with eluent systems of dichloromethane - methanol - benzene (25:1:0.5; 25:4:3 and 25:6:3, v/v/v). In the second dimension toluene - acetone - ethanol (96%) - ammonia (50:70:16:4.5, v/v/v/v) was used. White arrows show the fluorescent spot of dacryhainansterone in the chromatogram at 366 nm after spraying with vanillin - sulfuric acid.



The presence of herkesterone and 25-hydroxydacryhainansterone in the extract was investigated by derivative RP-HPLC using dual UV detection. Chromatograms of the extract at both 245 nm and 298 nm were determined, and the coordinates of the curves were translocated to Microsoft Excel as described in section **2.3.2**. The difference quotients of the neighbouring data points were calculated and, through plots of these quotients, good approximations of the first derivative curves could be obtained. **Fig. 15** shows the result of the analysis.

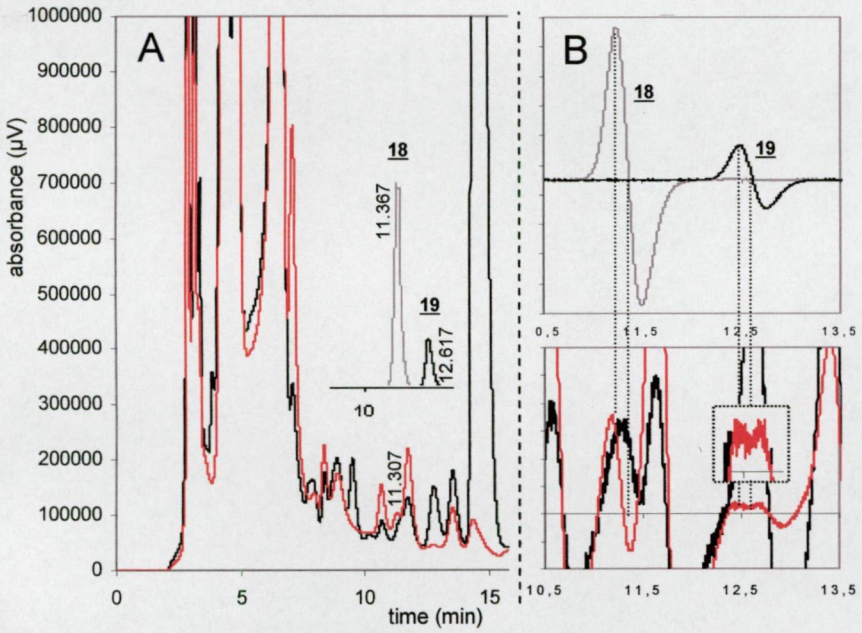


Figure 15. Examination of the presence of herkesterone (**18**) and 25-hydroxydacryhainansterone (**19**) in the extract of the plant.

Fig. 15A depicts RP-HPLC chromatograms of the extract at 245 nm (black) and 298 nm (red). The mobile phase was water - methanol (55:45, v/v) at a flow rate of 0.8 ml/min using column C7. Chromatograms of herkesterone and 25-hydroxydacryhainansterone are also presented. **Fig. 15B** shows the first derivative curves of each chromatogram, where the dashed box indicates the 10x zoom of the corresponding part of the curve below it.

Fig. 15A reveals a peak at 298 nm, which corresponds to that of herkesterone. Moreover, the derivative curves on **Fig. 15B** also show that the shape of the chromatogram changes if detection is carried out at 298 nm instead of 245 nm. The same inflection points appear as in the case of pure herkesterone and 25-hydroxydacryhainansterone, and a local minimum can also be observed in the derivative curve at 298 nm, which corresponds to the retention time of 25-hydroxydacryhainansterone. These facts suggest that both compounds may be present in the extract, and biosynthesized by the plant.

3.6. Structure elucidation of the ecdysteroids

Twenty-one known ecdysteroids were isolated, and were characterized by direct comparison of their physical and spectroscopic characteristics with those published in the literature [**104**]. They were also identified by co-chromatography with pure reference ecdysteroids, using NP- and/or RP-TLC and also NP- and/or RP-HPLC. Four new natural ecdysteroids were isolated [**128**,**129**] and three new ecdysteroids were additionally obtained by elimination of the 11α-OH group. Besides chromatography, all compounds were characterized by different spectroscopic methods (UV, IR, NMR and MS). MS and NMR provided the basic information on the structures of the compounds. In the course of the elucidation of the structures of the compounds, MS and NMR spectral data were usually evaluated in comparison with the those of the main ecdysteroid, 20E.

3.6.1. Chromatographic identification and physical properties

In the case of TLC, three stationary phases and ten mobile phases with different selectivities were used [**130**]. **Table 4** lists the TLC characteristics of the isolated ecdysteroids.

Table 4 TLC retention factors and detection of isolated ecdysteroids

Ecdysteroid	Number of (free) OH groups	Colour of spots		NP-TLC				RP-TLC						CN-TLC
		UV (366 nm)	daylight	mobile phases										
				1	2	3	4	5	6	7	8	9	10	11
Ajugasterone C **11**	6	dark-red	dark-red	35	-	34	35	45	29	34	-	47	35	24
Ajugasterone C 20,22-monoacetonide **3**	3	dark-red	dark-red	-	-	46	70	63	-	-	-	-	-	-
Ajugasterone C 2,3;20,22-diacetonide **1**	1	dark-red	dark-red	-	-	90	91	-	-	-	-	-	-	-
Dacryhainansterone **6**	5	red	brown	48	-	55	63	71	10	21	-	34	50	15
22-Desoxy-20E **12**	5	orange	orange	26	23	30	34	47	30	34	32	58	31	31
E **14**	5	violet	light-yellow	33	27	35	40	41	33	38	35	61	30	30
3-Epi-20E **22**	6	violet	turquise	-	-	27	30	38	-	-	-	-	-	-
Herkesterone **18**	7	red	red	26	-	-	25	-	57	54	-	68	21	51
5α-20E **13**	6	violet	turquoise	28	23	32	38	38	49	54	50	65	21	46

20E **17**	6	violet	turquoise	24	21	27	30	37	47	56	46	66	26	48
20E 2,3;20,22-diacetonide **2**	1	violet	turquise	-	-	81	86	89	-	-	-	-	-	-
11α-hydroxypoststerone **10**	3	turquise	light-red	-	-	21	30	40	-	-	-	-	-	-
Isovitexirone **20**	6	violet	dark-green	32	-	37	37	43	39	41	-	50	33	23
Makisterone A **15**	6	violet	purple	-	-	31	38	40	57	49	41	-	28	37
Makisterone C **9**	6	violet	violet	50	-	41	40	57	26	33	30	45	30	27
25-hydroxydacryhainansterone **19**	6	red	red	21	-	27	28	37	52	54	-	68	24	46
Polypodine B **4**	7	violet	turquoise	32	20	25	35	38	48	55	49	66	33	50
Pterosterone **7**	6	violet	green	-	-	-	49	59	-	-	-	-	-	-
Turkesterone **24**	7	dark-red	dark-red	10	-	12	14	19	99	68	-	77	8	62

Based on the above results, 2D-TLC screening of ecdysteroid pattern of *Serratula* species can also be purposed [**131**].

In the case of HPLC, mainly two NP-HPLC systems (NP-HPLC$_2$ and NP-HPLC$_6$) and one RP-HPLC system (RP-HPLC$_1$) were used for purification and purity control; the HPLC data of ecdysteroids are given according to these systems.

Table 5. Some HPLC data on the isolated and semisynthesized (denoted **) ecdysteroids *k'* = capacity factor and *α* = selectivity factor for 20E. New natural compounds are denoted *. Columns and mobile phases are given in section **3.11**

	k'	*α*	*k'*	*α*	*k'*	*α*
Ecdysteroid **Mobile phase >**	**NP-HPLC$_2^{C4}$**		**NP-HPLC$_6^{C4}$**		**RP-HPLC$_1^{C5}$**	
Ajugasterone C **11**	2.85	1.60	8.40	1.27	6.82	7.66
Ajugasterone C 20,22-monoacetonide **3**	0.64	7.14	-	-	-	-
Dacryhainansterone **6**	1.14	4.01	1.90	3.47	10.49	11.79
25,26-Didehydrodacryhainansterone(**)	-	-	2.68	2.46	8.29	9.31
9,11-Didehydropoststerone(**)	1.46	3.13	-	-	-	-
20,26-Dihydroxyecdysone **25**	14.68	3.21	15.41	2.34	0.84	1.06
22-Desoxy-20E **12**	3,64	1,26	5,2	1,27	3,49	3,92
E **14**	3.29	1.39	4.66	1.41	3.55	3.99
3-Epi-20E **22**	3.93	1.16	6.55	1.01	-	-
14-Epi-20E(*) **21**	4.14	1.10	6.62	1.01	-	-
22-Epi-20E **23**	8.43	1.84	7.89	1.19	-	-
Herkesterone(*) **18**	3.46	1.32	-	-	0.55	1.62
25-Hydroxydacryhainansterone(*)(**) **19**	4.61	1.01	8.00	1.21	0.58	1.53
5α-20E **13**	3.39	1.34	7.28	1.10	0.66	1.35
20E **17**	4.57	1	6.59	1	0.89	1
11α-Hydroxypoststerone(*) **10**	2.82	1.62	6.7	1.01	0.67	1.33
Isovitexirone **20**	3.04	1.50	4.14	1.59	2.33	2.62
Makisterone A **15**	3.54	1.29	4.86	1.36	1.39	1.56
Makisterone C **9**	1.79	2.55	3.48	1.89	6.03	6.78
Polypodine B **4**	3.29	1.39	6.72	1.02	0.98	1.03
Pterosterone **7**	4.25	1.08	-	-	2.42	2.83
Turkesterone **24**	8.46	1.85	11.4	1.73	-	-
Mobile phase >			**RP-HPLC$_2^{C5}$**		**RP-HPLC$_4^{C5}$**	
Dacryhainansterone 2,3;20,22-diacetonide(**)			-	-	7.42	-
Ajugasterone C 2,3;20,22-diacetonide **1**			11.80	-	2.23	-
20E 2,3;20,22-diacetonide **2**			9.06	-	-	-

The IR and UV spectra yielded characteristic information on the 7-en-6-one chromophore. Typically, the ecdysteroids are characterized by a strong UV absorption

spectrum centred at 242 nm (log $\varepsilon \approx 4$). The structural differences between the compounds are minor, and in the regions distant from the chromophore they were essentially indistinguishable from each other. Infrared spectroscopy demonstrated a C=O band at 1640 cm^{-1}, and a C=C signal at 1612 cm^{-1}. In the IR spectrum, a wide absorption band at 3300-3500 cm^{-1} reveales the presence of hydroxy groups. The fingerprint regions indicated some differences between the compounds, enabling them to be distinguished from each other. **Table 6** presents physical and spectroscopic data on the compounds, such as melting point, results of optical rotation and/or circular dichroism measurements, and IR and UV spectroscopic data.

Table 6. Physical, IR and UV spectroscopic data on ecdysteroids from *S. wolffii*

Ecdysteroid	M.P.(C°)	$[\alpha]_D^{20}$ (*c*; MeOH)	IR (KBr) νmax cm–1	UV λmax nm (log ε)
Ajugasterone C **11**	216–217	$[\alpha]_D^{18}$ +48,5° (*c* 1.1)	3400 (OH), 1655 (7-en-6-one)	243 (4.014)
Ajugasterone C 2,3;20,22-diacetonide **1**	–	$[\alpha]_D^{31}$ +33±2° (*c* 0.1)	3410 (OH), 1670 (7-en-6-one), 1380, 1373, 1059	241,3 (4.028)
Ajugasterone D **5**	234–237	$[\alpha]_D^{20}$ + 55.0 ° (*c* 0.18)	3400 (OH),1690 (7-en-6-one)	244 (4.07)
Dacryhainansterone **6**	–	$[\alpha]_D^{27}$ +51° (*c* 2.25)	–	298 (4.152), 235 (3.806)
22-Deoxy-20,21-dihydroxyecdysone **16**	–	$[\alpha]_D^{26}$ + 78° (*c* 0.1)	3420 (OH), 1641 (7-en-6-one)	242
22-Deoxy-20E **12**	241-242	$[\alpha]_D^{20}$ + 80.9 ± 2° (*c* 0.42)	3400-3430 (OH), 1710, 1647 (7-en-6-one)	244 (4.03)
20,26-DihydroxyE **25**	149-153	$[\alpha]_D^{28}$ +26° (*c* 0.5)	3486 (OH), 1652 (7-en-6-one)	242 (4.041)
E **14**	237-239	$[\alpha]_D^{20}$ + 63.6 ° (*c* 0.83)	3333 (OH), 1657 (7-en-6-one)	242 (4.093)
3-Epi-20E **22**	215–216	$[\alpha]_D^{15}$ +61,5° (*c* 0.88)	3410 (OH) 1653 (7-en-6-one)	241 (4.093)
14-Epi-20E **21**	–	$[\alpha]_D^{27}$ -4° (*c* 1)	3380 (OH), 1653 (7-en-6-one), 1063	247.9 (3.616)
22-Epi-20E **23**	259–260		1650 (7-en-6-one)	
Herkesterone(*) **18**	218	$[\alpha]_D^{28}$ +59° (*c* 0.1) DMSO	3560–3200 (OH), 1650 (7-en-6-one), 1602	296 (4.02)
25-Hydroxydacryhainansterone(*) **19**	–	$[\alpha]_D^{29}$ -17° (*c* 0.5)	–	297.8 (3.334)
5α-20E **13**	278	$[\alpha]_D^{20}$ + 55.5 ° (*c* 0.54)	3400 (OH), 1655 (7-en-6-one)	242 (-)
20E **17**	241-242.5	$[\alpha]_D^{20}$ + 58.9 ± 2° (*c* 0.3)	3500 (OH), 1645 (7-en-6-one)	240 (4.103)
20E 2,3;20,22-diacetonide **2**		$[\alpha]_D^{31}$ +35±2° (*c* 0.1)	3449 (OH), 1664 (c-hexenone), 1381, 1373, 1169, 1059 (dioxolane ring)	242.8 (4.035)
20E 20,22-monoacetonide **8**	227–229	$[\alpha]_D^{23}$ +60,1±2° (*c* 1.3)	3400–3465 (OH), 1660 (7-en-6-one). 1384,1371, 1161, 1052	243 (4.01)
11α-Hydroxypoststerone **10**	174–176	$[\alpha]_D^{28}$ +12° (*c* 0.1)	3320, 1718, 1653 (7-en-6-one)	240 (4.116)
Isovitexirone **20**	–	$[\alpha]_D^{27}$ +31±2° (*c* 2.2)	3391 (OH), 1653 (7-en-6-one) 1581 extra C=C	241.5 (3.880)
Makisterone A **15**	263–265	$[\alpha]_D^{24}$+60,3±2° (dioxane)	3420 (OH), 1655 (7-en-6-one), 1630	243 (4.09)
Makisterone C **9**	258-259	$[\alpha]_D^{25}$ + 54.82 ± 2° (*c* 1.3)	3400-3470 (OH) 1643-1650 (7-en-6-one)	243-244 (1.146)
Polypodine B **4**	254-257	$[\alpha]_D^{26}$ + 94.2 ± 2° (*c* 0.05)	3400 (OH), 1673 (7-en-6-one)	243 (4.11), 317 (2.07)
Pterosterone **7**	229–230	$[\alpha]_D^{20}$+7,4°	3420 (OH), 1650 (7-en-6-one)	243
Turkesterone **24**	–	$[\alpha]_D^{29}$ +4 ±2° (*c* 1)	3300–3500 (OH), 1660 (7-en-6-one)	244 (3.95)
25,26-Didehydrodacryhainansterone(**)	–	$[\alpha]_D^{29}$ +30 ±2° (*c* 1)	-	298 (3.786); 224
9,11-Didehydropoststerone(**)	–	$[\alpha]_D^{29}$ -5 ±2° (*c* 1)	-	295.7; (3.374); 231

3.6.2 Mass spectrometry

For the determination of molecular mass, CIMS and ESIMS were used. EIMS gave molecular ions of low intensity. The mass spectra of ecdysteroids may be characterized by the appearance of numerous signals, differing from each other by the loss of water (18 units) from the polyhydroxylated ecdysteroids. Moreover, the majority of ecdysteroids suffer side-chain cleavage; the splitting occurs between C-20 and C-22, and C-17 and C-20. The mass spectra are characterized by mass numbers which depend on the degrees of hydroxylation of the side-chain and nucleus. Fragmentation may result in two major series derived from the nucleus or side-chain, as shown in **Table 7**. Further cleavage may occur between C-22 and C-23, C-23 and C-24, or C-24 and C-25. In ring D, either C-13−C-17 or C14−C-15 fragmentation may take place.

Table 7. MS fragmentation of the isolated ecdysteroids

Ecdysteroid	M_R	Method	Mean MS fragments m/z (relative intensity %)
Ajugasterone C **11**	480	EIMS	462 $[M-H_2O]^+$, 379 (5), 361 (16), 343 (64), 335 (1), 325 (39), 317 (2), 299 (6), 281 (4), 145 (23), 127 (6), 109 (21), 101 (2), 83 (25), 43 (100)
		FABMS	480 $[M]^+$ (12)
		DEI	481 $[M+H]^+$ (0,1), 480 $[M]^+$ (0,1), 343 (100)
Ajugasterone C 2,3;20,22-diacetonide **1**	560	FABMS	583 $[M+Na]^+$ (20), 561 $[M+H]^+$ (20), 543 (25), 485 $[M+H-H_2O\text{-acetone}]^+$ (28), 427 (10)
Ajugasterone D **5**	478	EIMS	479 $[M+H]^+$, 443, 379 $[M-C_{22}-H_{27}]^+$, 361, 343, 325, 143 $[C_{20}-C_{27}]^+$, 125 $[C_{22}-C_{27}]^+$, 81
Dacryhainansterone **6**	462	CIMS	480 $[M+NH_4]^+$ (11), 463,6 $[M+H]^+$ (100), 447,6 $[M-CH_3]^+$ (51), 445,6 $[M+H-H_2O]^+$ (32), 427 $[M+H-2H_2O]^+$ (21), 362,5 (74), 345,5 $[M-C_{20}-C_{27}]^+$ (61)
22-Deoxy-20E **12**	464	EIMS	464 $[M]^+$ (0.3), 446 (1.4), 431 (0.8), 428 $[M-2\ H_2O]^+$ (35), 418 (1), 413 (16), 410 (28), 400 (2.5), 395 (12), 385 (0.7), 343 (5), 327 (54), 325 (20), 320 (20), 309 (10), 302, 301 (19), 300 (35), 299 (27), 250 (30), 249 (15), 145 (20), 127 (97), 109 (100), 81 (20), 69 (54).
20,26-Dihydroxyecdysone **25**	496	CIMS	514 $[M+H+NH_3]^+$, 497 $[M+H]^+$,479,461,443,380, 363 $[MH-C_{22}-C_{27}]^+$, 347, 345, 329
E **14**	464	EIMS	446 $[M-H_2O]^+$ (3), 428 $[M-2H_2O]^+$ (29), 413 (2), 410 $[M-3H_2O]^+$ (3), 359 (4), 348 (8), 330 (15), 300 (23), 99 $[C_{22}-C_{27}]^+$ (100), 81 (61).
3-Epi-20E **22**	480	EIMS	480 $[M]^+$, 462 $[M-18]^+$ (<1), 444 (1), 426 (16), 411 (10), 408 (6), 393 (4), 375 (2), 363 (4), 352 (10), 345 (45), 344 (17), 329 (17), 328 (38), 327 (49), 313 (11), 311 (10), 310 (14), 309 (17), 301 (20), 300 (33), 285 (31), 269 (21), 267 (21), 173 (26), 99 (100), 81 (62)
		CIMS	481 $[M+H]^+$, 463, 445, 427, 363, 345
14-Epi-20E **21**	480	FABMS	503 $[M+Na]^+$ (34), 481 $[M+H]^+$ (41), 463 $[M+H-H_2O]^+$ (25), 445 $[M+H-2H_2O]^+$ (65), 427 $[M+H-3H_2O]^+$ (37), 363 (41), 303 (71), 279 (90), 211 (100)
22-Epi-20E **23**	480	CIMS	498 $[M+H+NH_3]^+$, 481 $[M+H]^+$, 463 $[M+H-H_2O]^+$, 445 $[M+H-2H_2O]^+$, 427 $[M+H-3H_2O]^+$, 409 $[M+H-4H_2O]^+$, 380 $[M+NH_3-C_{22}-C_{27}]^+$, 363 $[M-C_{22}-C_{27}]^+$, 345
Herkesterone **18**	494	ESIMS	495 $[M+H]^+$ (9), 477 $[M+H-H_2O]^+$ (10), 459 (23), 443 (26), 440 (11), 422 (8), 407 (49), 394 (17), 378 (9), 361 (26), 359 (10), 323 (18), 300 (100), 199 (11)
5α-20E **13**	480	ESIMS	503 $[M+Na]^+$ (2), 463 $[M-H_2O+H]^+$ (18), 445 $[M-2H_2O+H]^+$ (10), 413 (1), 391 (2), 301 (2), 279 (1), 247 (20), 223 (3), 97 (100), 87 (23), 65 (22).
20E **17**	480	CIMS	498 $[M+H+NH_3]^+$, 481 $[M+H]^+$, 463, 445, 427, 380, 363, 347, 345, 329.
		EIMS	480 $[M]^+$ (<1), 462 $[M-H_2O]^+$ (1), 444$[M-2H_2O]^+$ (1), 429 (3), 426 $[M-3H_2O]^+$ (12), 411 (2), 408 $[M-4H_2O]^+$ (3), 393 (1), 363 $[M-C_{22}-C_{27}]^+$ (7), 346 (11), 345 $[M-H_2O-C_{22}-C_{27}]^+$ (30), 344 (26), 328 (17), 327 (19), 300 (13), 145 (8), 143 $[C_{20}-C_{27}-H_2O]^+$ (8), 99 $[C_{22}-C_{27}]^+$ (100), 81 $[C_{22}-C_{27}-H_2O]^+$ (27).
		ESIMS	503 $[M+Na]^+$ (20), 481$[M+H]^+$ (50), 463 $[M-H_2O+H]^+$ (100), 445 $[M-2H_2O+H]^+$ (10), 413 (1), 391 (2), 301 (2), 279 (1), 251 (6), 247 (20), 223 (3), 215 (2), 119 (5), 97 (100), 87 (23), 65 (22).
25-Hydroxydacryhainansterone **19**	478	CIMS	496,7 $[M+NH_4]^+$ (1), 479 $[M+H]^+$ (9), 463,6 $[M-CH_3]^+$ (15), 461,6 $[M+H-H_2O]^+$ (12), 445 $[M-CH_3-H_2O]^+$ (6) 427 $[M+H-3H_2O]^+$ (8), 362,5 (64), 345,5 $[M-C_{20}-C_{27}]^+$
20E 2,3;20,22-diacetonide **2**	560	EIMS	560 (1), 545 (12), 527 (10), 509 (5), 403 (100), 385 (25), 341 (20)
20E 20,22-monoacetonide **8**	520	EIMS	520 $[M]^+$, 505 (2), 502 (1), 487 (4), 469 (5), 445 (4), 427 (29), 409 (13), 363 (100), 353 (15), 345 (26), 329 (10), 327 (9), 320 (4), 300 (32), 201 (13), 143 (11), 99 (19), 81 (26)

11α-Hydroxypoststerone **10**	378	FABMS	379 $[M+H]^+$ (100), 361 $[M+H-H_2O]^+$ (88), 343 $[M+H-2H_2O]^+$ (40), 325 $[M+H-3H_2O]^+$ (10), 299 (11), 282 (32), 277 (13), 249 (80), 231 (20)
Isovitexirone **20**	478	FABMS	501 $[M+Na]^+$ (56), 479 $[M+H]^+$ (65), 461 (100), 443 (22), 278 (56)
		EIMS	478 (0), 461 (1), 442 (2), 426 (2), 408 (1), 393 (1), 379 (1), 361 (7), 360 (8), 343 (28), 325 (15), 267 (20), 171 (25), 69 (37), 55 (42), 43 (100), 41 (70)
Makisterone A **15**	494	EIMS	494 $[M]^+$, 363 (100), 345 (78), 131 (24), 113 (59), 95 (30), 70
Makisterone C **9**	508	EIMS	508 $[M]^+$, 493, 490, 475, 472, 457, 454, 439, 363, 345, 189, 171, 145 $[M-363]^+$, 127, 109
Polypodine B **4**	496	EIMS	478 $[M-H_2O]^+$, 460, 442, 424, 361, 360, 344, 343, 99, 81.
Pterosterone **7**	464	EIMS	480, 462, 444, 426, 408, 363 $[M-C_{22}-C_{27}]^+$, 345, 328, 117, 99, 81
Turkesterone **24**	496	EIMS	460 $[M-2H_2O]^+$, 442, 424, 409, 379, 361, 343, 325, 300, 143

3.6.3. NMR spectroscopy

From the ^{13}C NMR, DEPT and HMQC spectra, the numbers of C, CH, CH_2 and CH_3 fragments in the molecule were first identified. From the ^{13}C chemical shifts (> 60 ppm), the numbers of connecting oxygen atoms were established. The methyl groups could be utilized as starting point for the determination of the structure, because their signals are singlets and display strong two- and three-bond correlations in the HMBC spectrum. In the knowledge of these HMBC correlations and of the ecdysteroid skeleton, methyl groups could be unambiguously identified in positions 18, 19, 21, 26 and 27 (the signals of H-26 and H-27 permitted assignment of the corresponding carbon atoms, while those of H-18 and H-21 led to the identification of C-17). Protons of sp^2 carbon atoms gave correlations to C-5, C-9 and C-14 in the HMBC spectra, which proved the 7,8 double bonds in all compounds. The NOESY correlations provided information about the stereochemistry of the rings and the orientation of the substituents connected to the skeleton. It is important to determine the anellation of rings A/B, which can be *cis* or *trans*. NOESY signals correlating H-9 with H-1 and H-5, and H-19 with H-4, are due to *trans*-A/B ring junction. However, the correlations between H-9/H-2, H-9/H-4 and H-19/H-5 prove that rings A and B are *cis*-fused (**Fig. 14**).

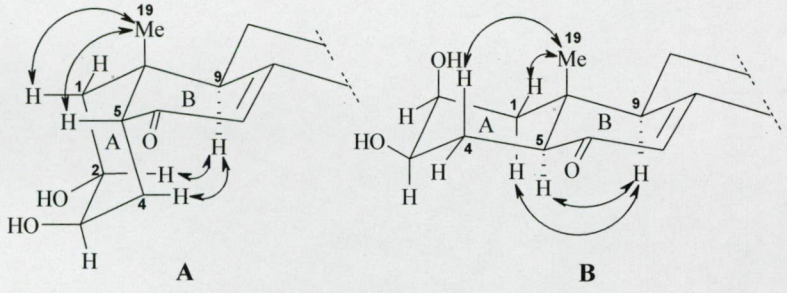


Figure 14. Selected NOESY correlations for structure **A** (A/B *trans*) and **B** (A/B *cis*) (*e.g.*, 20E (**17**) and 5α-20E (**13**), respectively). Double arrows indicate the characteristic NOESY correlations.

It is easier to give specific modifications in the ^1H-NMR spectra with reference to the E and 20E molecules, which can be utilized for structure assessment. From the analysis of the compounds 1D and 2D ^1H-NMR data, it is clear that the steroid nucleus of the compounds is

'classical' with respect to the presence of the 3β-OH, 14α-OH, and 2β-OH functionalities (except in the cases of 3-epi-20E (**22**) and 14-epi-20E (**21**)).

(a) Compounds in the E versus 20E series could be easily distinguished by the H-21 signal, which undergoes changes both in its chemical shift and in shape: a singlet for 20-OH compounds, and a doublet in 20-deoxy compounds.

(b) The presence of an α,β,γ-conjugated ketone was established on the basis of HMBC correlation of H-7 with the quaternary =C signal and further supported by the H-11/H_2-12 correlations observed in the ^{1}H, ^{1}H-COSY spectrum.

(c) The presence of a 7,9(11)-dien structure was also established on the basis of the HMBC spectra as in herkesterone (**18**): H-7 (5.83 ppm) showed a correlation with the quaternary sp^2 carbon (C-9) at δ 137.9 ppm, and a correlation could be observed in the ^{1}H,^{1}H-COSY spectra between H-11 and H_2-12.

(d) The presence of an α-OH-group on C-11 as in ajugasterone C (**11**) was proved by the shifts in the H-11 and C-11 signals and by the multiplicity of the H-11 signal.

(e) 22-Deoxyecdysteroids exhibit characteristics similar to those of 22-deoxy-20E (**12**): the lack of the H-22 signal in the hydroxymethine zone, a small downfield shift of H-21 (+ 0.08-0.1 ppm in MeOH-*d*4), and small upfield shifts of H-18 and H-17 (− 0.04-0.08 ppm in MeOH-*d*4).

(f) 5β-Hydroxyecdysteroids, *e.g.* turkesterone (**24**), show the disappearance of the H-5 signal, a modification of the H_{ax}-2 and H_{eq}-3 signals (which become nearly isochronous and give a complex multiplet at 3.94-3.99 ppm in MeOH-*d*4), signal broadening at 4.15-4.26 ppm in C_5D_5N, a small upfield shift of the 19-methyl signal (− 0.07 ppm in MeOH-*d*4), a large downfield shift of the H_{ax} signal (resulting from the axial interaction between 5β-OH and H_{ax}-1, as observed in the ^{1}H,^{1}H-COSY experiments), and a large downfield shift of the H_{ax}-4 and H_{eq}-4 signals.

(g) 26-Hydroxyecdysteroids, *e.g.* 20,26-dihydroxyecdysone (**25**), are characterized by the appearance of a hydroxymethyl signal (two if the 25*R* and 25*S* diastereoisomers are present) as a singlet at δ = 3.35 ppm and the loss of the H-26 signal, and an upfield shift of the H-27 signal (- 0.05 ppm in MeOH-*d*4).

(h) 5α-Ecdysteroids (A/B *cis*-fused) reveal upfield shifts for H_{ax}-9 (− 0.43 ppm) and H_{ax}-3 (− 0.37 ppm) (in MeOH-*d*4), while H-2 is equatorial (width at half height, $w_{1/2}$ = 8 Hz) and H-3 is axial ($w_{1/2}$ = 22 Hz). The 5α-configuration is confirmed by the strong NOE between H-9 and H-5 and H_{ax}-1 (see above).

(i) The C_{28} and C_{29} ecdysteroids usually contain one or two alkyl groups at C-24 (*e.g.* C_{28}: makisterone A [24*R*] (**15**), and C_{29}: makisterone C (**9**)). Their NMR spectra are characterized by the appearance of either 28-Me (14.9 ppm at C-28; 0.98 ppm (*d* 6.9)) or 28- and 29-Me signals (1.14, 1.53 at H-28*a*, *b*; 1.01 at H-29), as in **15** and **9**, respectively.

(j) A furan ring may be generated by cyclization of a parent C_{27} skeletal side-chain through dehydration of the C-22 and C-25 hydroxy groups as in ajugasterone D (**5**). In this case, the most significantly shifted are C-22 and C-25, which move downfield 7.2 and 10.5 ppm, respectively, as compared to 20E. These shifts are those expected as the two hydroxyl groups on the carbons in 20E are dehydrated to form an ether linkage (C-22-O-C-25). The connectivities within the five-membered ring are confirmed by NOESY signals, such as the correlation of H-22 with H-26 during cyclization of the side-chain, and the proton on C-25 must therefore be in the vicinity of H-22.

(k) The 3α-OH group of 3-epi-20E (**22**) was identified via the ^{1}H and HMQC spectra: the multiplicity of H-3 is a triplet ($J > 8$ Hz), in place of the usual quadruplet ($J \approx 3$ Hz), which together with the H-3/H-5 NOESY cross-peak proves that 3-OH is in an α and equatorial position.

(l) The presence of the 14α-OH group (and *cis* anellation of rings C/D) in 14-epi-20E (**21**) was proved by the NOESY correlations between H-9/Hα-15, Hα-12/Hα-15 and Hα-17/Hβ-18

(m) The acetonide group in position 20,22, as in ajugasterone C 20,22-acetonide (**3**), was identified through the ^{13}C spectra: the chemical shifts of C-20 and C-22 (which can be assigned by H_3-21) appear at 82.01 and 84.88 Hz instead of the value for ajugasterone C near 76.9 ppm. Two extra methyl-group could also be seen connected to a typical (O-C-O) sp^3 quaternary carbon atom with a chemical shift of 106.92 ppm. Identification of 2,3-acetonide groups as in ajugasterone C 2,3;20,22-diacetonide (**1**) was accomplished in the same way using the chemical shifts of C-2 and C-3.

Tables 8-11 furnish the ^{1}H (500 MHz) and ^{13}C (125 MHz) chemical shifts (in ppm), $J(^1H, ^1H)$ couplings (in Hz), 2D ^{1}H,^{1}H scalar couplings (COSY) characteristic ^{13}C,^{1}H long-range correlations (HMBC), spatial proximities (NOESY), and individual spin system (TOCSY) data on the new ecdysteroids isolated(*) and the semisynthesized compounds(**).

Table 8. NMR data on 14-epi-20E (*) (**21**) and 25-hydroxydacryhainansterone (*) (**19**)

Multiplicity of signals: s, singlet ; d, doublet; t, triplet; q, quadruplet; m, multiplet

14-Epi-20E							**25-Hydroxydacryhainansterone**						
No.	**^{13}C**	**^{1}H**	***m*; *J* (Hz)**	**NOESY**	**HMBC**	**HMQC-TOCSY (TOCSY only)**		**^{13}C**	**^{1}H**	***m*; *J* (Hz)**	**COSY**	**HMBC**	**NOESY**
1 α	37.4	1.79				3.94, 3.86	**1** α	37.5	2.08	dd; 13.5, 4.0	3.72	36.9	6.29, 3.72, 1.10
β		1.44					β		1.70	dd; 13.5, 11.9	3.72, 1.81	-	3.72, 2.45, 1.10
2 α	68.8	3.86	dt; 11.9, 3.7	3.94, 2.85, 1.80, 1.60, 1.44	-	1.80	**2** α	68.9	3.72	ddd; 11.9, 3.9, 3.1	3.85, 2.08, 1.70	-	3.85, 2.08, 1.70, 1.60
3 α	68.6	3.94	s, broad	3.86, 1.73, 1.60	-	1.73	**3** α	68.4	3.85	q; 2.8	3.73, 1.76, 1.61	-	3.72, 1.76, 1.61
4 α	33.2	1.60				3.94, 2.36	**4** α		1.60				
β		1.73					β		1.76				
5 β	51.2	2.36	dd; 13.2, 4.0	1.73, 1.44, 0.95	206.3	1.72, 1.60	**5** β	51.7	2.45	dd; 12.6, 3.7	1.76, 1.60	-	1.74, 1.70, 1.10
6	206.3	-	-	-	-	-	**6**						
7	123.3	6.29	d; 2.2		85.8, 51.2, 37.6	-	**7**	119.4	5.75	d; 1.0	6.29, 2.74	136.6, 84.6, 51.7	1.95, 1.79
8		-	-	-	-	-	**8**	156.7	-	-	-	-	-
9 α	37.6	2.85	s, very broad	3.86, 2.28, 1.79, 1.60	-	1.69	**9**	136.3	-	-	-	-	-
10	39.4	-	-	-	-	-	**10**	41.1	-	-	-	-	-
11 α	22.4	1.78				2.85	**11**	134.0	-	-	-	-	-
β		1.67											
12 α	41.8	1.76				2.85, 2.41	**12** α	39.2	2.735	dd; 17.6, 2.5	6.29, 5.75, 0.90	136.2, 134.0, 18.3	6.29, 2.49, 1.211
β		1.68					β		2.42	dd; 18.0, 6.8	6.29	136.2, 134.0, 84.6	6.29, 1.21, 0.90
13	52.8	-	-	-	-	-	**13**	48.1	-	-	-	-	-
14	85.8	-	-	-	-	-	**14**	84.6	-	-	-	-	-
15 α	40.9	2.28	td; 11.9, 8.7	2.86, 1.89, 1.77	-	1.98, 1.89	**15** α		1.79				
β		1.43					β		1.95				
16 α	24.8	1.89	dt; 12.5, 9.0	3.50, 1.98	85.8	1.98, 1.43	**16** α	22.0	1.80				
β		2.09		3.50, 1.43	56.8		β		1.99				
17 α	56.8	1.98	dd; 9.0, 7.4	3.50, 1.89, 1.77, 1.69, 1.58, 1.29	85.8, 52.8, 41.8, 24.8	-	**17** α	50.7	2.495	t; 8.9	1.99; 1.80	48.0, 22.0, 18.3	2.74, 1.78, 1.67, 1.211
18 β	19.4	1.26	s; overlapped	1.68	85.8, 56.8, 52.8, 41.8	-	**18** β	18.3	0.90	s	2.73	84.7, 50.7, 48.1, 39.3	2.45, 2.42, 2.01, 1.96
19 β	24.8	0.945	s	6.29, 2.37, 1.79, 1.68, 1.45	51.2, 39.4, 37.6, 37.4	-	**19** β	31.6	1.106	s	-	136.3, 51.7, 41.1, 37.5	
20	77.8	-	-	-	-	-	**20**	78.0	-	-	-	-	-
21	20.6	1.29	s; overlapped	1.98	78.6, 77.8, 56.8	-	**21**	21.0	1.211	s	-	78.6, 78.0, 50.7	2.74, 2.45, 2.42, 1.64
22	78.6	3.50	d; 10.3	2.09 , 1.98, 1.89, 1.59, 1.42, 1.31	77.8, 20.6	1.80, 1.43	**22**	78.6	3.34	overlapped	1.64, 1.31	-	too much T_1-noise
23 *a*	27.6	1.27				3.50,	**23** *a*		1.31				
b		1.59					*b*		1.64				
24 *a*	42.3	1.41				3.50, 1.59	**24** *a*	42.5	1.44	ddd; 13.6, 11.5, 4.3	1.66, 1.31	-	3.34, 1.69
b		1.80					*b*		1.81				
25	71.4	-	-	-	-	-	**25**	71.6	-	-	-	-	-
26	29.0	1.19	s	1.81, 1.59	71.3, 42.3, 30.0	-	**26**	29.1	1.19	s	-	71.6, 42.5, 29.8	1.81, 1.70, 1.64
27	30.0	1.21	s	1.81, 1.59	71.3, 42.3, 29.0	-	**27**	29.8	1.206	s	-	71.6, 42.5, 29.1	

Table 9. NMR data on herkesterone(*) (**18**)

Because of the small amount of sample, the NMR spectra had to be measured in both MeOH-d_4 and dimethylsulfoxide-d_6 (DMSO) in order to obtain satisfactory evidence for the suggested structure. The differences in the case of 20E are 5β-OH and the 9,11 olefin bond.

Methanol						DMSO					
No.	^{13}C	^{1}H	m; J (Hz)	HMBC	NOESY	No.	^{13}C	^{1}H	m; J (Hz)	HMBC	NOESY
1 α	34.5	2.05		137.9, 70.3, 68.6, 46.4	6.34, 3.83, 1.05	1 α		1.87			
β		2.05		137.9, 70.3, 68.6, 46.4	1.05	β					
2 α	68.6	3.83	ddd; 10.7, 5.4, 3.2	-	6.34, 2.06, 1.92	2 α					
3 α	70.3	3.88	q; 3.0	-	3.83, 1.92, 1.77	3 α					
4 α	39.2	1.92	dd; 14.5, 2.8	-	3.88, 3.83	4 α					
β		1.77	dd; ?, 3.4	-	3.88	β					
5	80.5	-	-	-	-	5	78.4	-			
6		-	-	-	-	6					
7	117.9	5.83	t; 1.2	137.9, 84.5, 80.5	1.98, 1.80	7	116.5	5.65	s	136.3, 82.0, 78.4	1.89, 1.81, 1.71, 1.62
8	156.5	-	-	-	-	8					
9	137.9	-	-	-	-	9	136.3				
10	46.4	-	-	-	-	10					
11	134.3	6.34	dt; 6.7, 2.0	156.5, 48.0, 46.4	3.83, 2.73, 2.44, 1.05, 0.90	11	131.7	6.18	s; broad	-	3.59, 2.29, 1.87
12 α	39.2	2.726	dd; 18.0, 1.8	137.9, 134.3, 48.0, 18.2	6.34, 2.49, 1.208	12 α	37.5	2.59	d; 17.8	17.3	2.36
β		2.436	dd; 18.2, 6.7	137.9, 134.3, 84.5, 48.0, 18.2	6.34, 1.208, 0.90	β		2.29	dd; 17.8, 6.6	136.4, 131.7, 46.4, 17.3	6.18, 1.075, 0.77
13	48.0	-	-	-	-	13	46.3	-			
14	84.5	-	-	-	-	14	82.0	-			
15 α	31.5	1.80				15 α					
β		1.98				β					
16 α	21.8	1.79				16 α	20.4	1.60			
β		1.99				β		1.89			
17 α	50.7	2.49	t; 9.0	48.1, 21.8, 18.2	3.35, 2.726, 1.80, 1.68, 1.208	17 α	48.7	2.36	t; 9.3	46.2, 17.3	3.13, 2.59, 1.63, 1.49, 1.075
18 β	18.2	0.90	s	84.5, 50.7, 48.0, 39.2	6.34, 2.436, 1.98, 1.208, 1.05	18 β	17.3	0.77	s	82.0, 48.7, 46.3, 37.6	2.29, 1.90, 1.80, 1.075
19 β	26.5	1.05	s	137.9, 80.5, 46.4, 34.5	6.34, 2.05, 0.90	19 β	25.8	0.96	s; broad	-	-
20	77.8	-	-	-	-	20	75.5	-			
21	20.9	1.208	s	78.6, 77.8, 50.7	3.35, 2.73, 2.49, 2.44, 1.92-6	21	20.7	1.075	s	76.3, 75.5, 48.7	2.36, 2.29
22	78.6	3.35	dd; 10.3, 1.8	77.8, 42.5, 27.5, 20.9	2.49, 2.00, 1.79, 1.67, 1.44	22	76.3	3.13	d; 10.2	-	2.36, 1.89, 1.67, 1.62, 1.50, 1.27
23 *a*	27.5	1.30		78.6	1.80, ~1.2	23 *a*	26.1	1.13		-	1.67
b		1.68	dq; HMQC	-	3.35, 2.49, 1.44, ~1.2	*b*		1.49		-	3.13, 2.36, 1.09, 1.06
24 *a*	42.5	1.44	ddd; 13.3, 11.6, 4.2	71.4, 27.5	3.35, 1.68, ~1.2	24 *a*	41.4	1.27	td; 12.0, 4.3	68.7	3.13, 1.49
b		1.81	t; HMQC			*b*		1.65		68.7	
25	71.4	-	-	-	-	25	68.7	-			
26	29.1	1.19	s	71.4, 42.5, 29.9	1.80, 1.68, 1.44, 1.30	26	29.0	1.06	s	68.7, 41.4, 30.0	1.67, 1.49, 1.27
27	29.9	1.206	s	71.4, 42.5, 29.1	1.80, 1.68, 1.44, 1.30	27	30.0	1.09	s	68.7, 41.4, 29.0	1.67, 1.49, 1.27
						22-OH		4.13		-	1.07

		11α-Hydroxypoststerone					9,11-Didehydropoststerone				
No.		^{13}C	^{1}H	m; J (Hz)	HMBC	NOESY	^{13}C	^{1}H	m; J (Hz)	COSY	HMBC	NOESY
1	α	39.2	2.60	dd; 13.0, 4.2	69.1, 68.7, 53.0, 40.1	4.015, 3.18, 1.05	37.5	2.09	dd; 13.6, 4.0	3.725	41.1	6.34, 3.73, 1.10
	β		1.38	t; 12.3	69.1, 68.7, 53.0, 40.1, 24.8	2.345, 1.05		1.72	dd; 13.6, 11.9	3.725	136.7, 68.9	3.73, 2.46, 1.10
2	α	69.1	4.015	dt; 11.8, 3.8	-	2.60, 1.775, 3.96, 3.18	68.9	3.725	ddd; 11.9, 3.7, 3.4	3.86, 2.09, 1.72	-	6.34, 3.86, 2.09, 1.60
3	α	68.7	3.96	q; 2.9	-	4.015, 1.775, 1.70	68.4	3.86	td; 3.2, 2.7	3.725, 1.77, 1.61	-	3.725, 1.77, 1.60
4	α	33.5	1.775	td; 13.6, 2.4	53.0	3.96, 3.18	36.0	1.59		3.86, 2.46	-	3.86, 3.725
	β		1.70		-	3.96, 2.345		1.77	dt; 13.9, 3.9	3.86	-	3.86, 2.46
5	β	53.0	2.345	dd; 13.1, 4.1	206.6	1.70, 1.38, 1.05	51.7	2.46	dd; 12.8, 3.9	1.77, 1.59	-	1.77, 1.72, 1.10
6		206.6	-	-	-	-		-	-	-	-	-
7		123.3	5.807	d; 2.7	84.8, 53.0, 43.1	2.00, 1.68, 1.05, 0.61	120.0	5.77	s	6.34, 2.92	136.7, 84.4	1.99, 1.92
8		164.4	-	-	-	-	155.5	-	-	-	-	-
9	α	43.1	3.18	dd; 8.9, 2.7	164.4, 123.3, 69.4, 40.1, 24.8	4.08, 4.015, 2.60, 2.406, 1.775	136.7	-	-	-	-	-
10		40.1	-	-	-	-	41.1	-	-	-	-	-
11	β	69.4	4.08	ddd; 10.8, 8.9, 5.8	-	3.18, 2.08, 1.05, 0.61	133.2	6.34	dt; 6.6, 2.0	5.77, 2.92, 2.42	155.5, 48.3, 41.2	3.725, 2.93, 2.42, 2.09, 1.10, 0.63
12	α	42.3	2.406	t; 11.4	69.4, 48.6, 18.4	3.36, 3.18	37.2	2.926	18.0, 1.7	6.34, 5.77, 0.63	136.7, 133.2, 48.3, 17.8	6.34, 3.39, 2.17
	β		2.08	dd; 12.0, 5.8	84.8, 69.4, 48.6, 43.1, 18.4	4.08		2.42	17.8, 6.7	6.34	136.7, 133.2, 48.3, 17.8	6.34, 2.17, 0.63
13		48.6	-	-	-	-	48.3	-	-	-	-	-
14		84.8	-	-	-	-	84.4	-	-	-	-	-
15	α	32.3	1.68		84.8, 48.6	5.85, 1.90	31.9	1.92				
	α		2.00		22.4	5.85, 2.245, 0.61		1.99				
16	α	22.4	1.90		-	3.36, 1.68	22.6	1.925				
	β		2.245		212.3	2.00, 0.61		2.29	m	3.39, 2.01	60.1	0.63
17	α	60.0	3.36	dd; 9.4, 8.1	212.3, 22.4, 18.4	2.406, 2.16, 1.90	60.1	3.39	t; 8.6	2.29, 2.00, 1.92	48.4, 22.6, 17.9	2.92, 2.17, 1.95
18	β	18.4	0.61	s	84.8, 60.0, 48.6, 42.3	4.08, 2.245, 2.16, 2.00, 1.05	17.9	0.63	s	2.93	84.4, 60.1, 48.3, 37.2	6.34, 2.42, 2.29, 2.17, 2.00, 1.10
19	β	24.8	1.05	s	53.0, 43.1, 40.1, 39.2	4.08, 2.60, 2.345, 1.38, 0.61	31.7	1.10	s	-	136.7, 51.7, 37.5, 41.1	6.34, 2.46, 2.09, 1.72, 0.63
20		212.3	-	-	-	-	212.3	-	-	-	-	-
21		31.6	2.16	s	212.3, 60.0	3.36, 0.61	31.3	2.17	s	-	212.3, 60.1	3.39, 2.93, 2.42, 0.63

Table 10. NMR data on 11α-hydroxypoststerone(*) (**10**) and 9,11-didehydropoststerone(**)
These two ecdysteroids have a C_{21} skeleton (missing side-chain); 9,11-didehydropoststerone was derived from the semisynthetic transformation of **10**; appearance of a 9,11 olefine-bond and the lost of 11α-OH signals can be recognized (see above).

Table 11. NMR data on 24,25-didehydrodacryhainansterone(**) and dacryhainansterone 2,3;20,22-diacetonide(**)

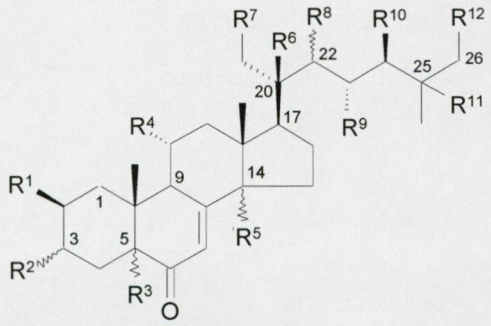
Semisynthesized from isovitexirone and ajugasterone C 2,3;20,22-diacetonide respectively; these two compounds show the lost of the 11α-OH group and the appearance of the 7,9(11)-diene structure.

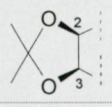
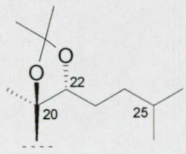
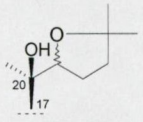
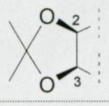
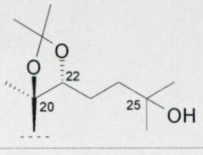
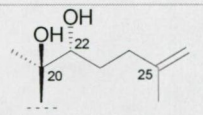
24,25-Didehydrodacryhainansterone							Dacryhainansterone 2,3;20,22-diacetonide						
No.	^{13}C	^{1}H	*m*; *J* (Hz)	HMBC	NOESY		No.	^{13}C	^{1}H	*m*; *J* (Hz)	HMBC	COSY	
1	α37.6	2.08					**1**	α 38.2	2.26	dd; 14.3, 5.8	73.7, 72.8, 52.2	4.20, 1.51	
	β	1.705	dd; 13.5, 11.8	136.3, 68.9, 41.0	1.10			β	1.51	dd; 14.4, 8.7	136.2, 73.5, 40.3	4.20, 2.26	
2	α68.9	3.715	ddd; 11.8, 3.9, 3.2	-	6.29, 3.84, 2.09, 1.59		**2**	α 73.4	4.20	dt; 8.5, 5.4	-	4.15, 2.26, 1.51	
3	α68.4	3.845	q; 3.0	-	3.72, 1.76, 1.59		**3**	α 72.9	4.15	q; 4.3	-	4.20, 1.97, 1.92	
4	α36.0	1.59	t; 13.3	-	3.84, 3.72		**4**	α	1.92				
	β	1.77						β	1.97				
5	β51.7	2.445	dd; 12.5, 4.1	-	1.69-1.79, 1.10		**5**	52.3	2.36	dd; 10.8, 4.7	-	1.94	
6							**6**		-	-	-	-	
7	119.3	5.75	d; 1.4	136.3, 84.7, 51.7	1.97, 1.80		**7**	119.3	5.76	d?; 0.7?	136.2, 84.7	6.22, 2.65	
8	157.0	-	-	-	-		**8**		-	-	-	-	
9	136.3	-	-	-	-		**9**	136.2	-	-	-	-	
10	41.0	-	-	-	-		**10**	40.2	-	-	-	-	
11	134.0	6.29	dt; 6.6, 2.0	157.0, 48.1, 41.0	3.72, 2.72, 2.41, 2.08, 1.10		**11**	134.1	6.22	dt; 6.5, 2.1	47.9, 40.4	2.65, 2.40	
12	α39.2	2.725	dd; 18.2, 2.0	136.3, 134.0, 48.1, 18.3	6.29, 2.47, 1.20		**12**	α 39.1	2.395	dd; 18.2, 6.6	136.4, 134.2, 84.5	6.22, 2.65	
	β	2.415	dd; 18.0, 6.7	136.3, 134.0, 84.7, 48.1	6.29, 1.20, 0.90			β	2.655	dd; 18.0, 2.2	18.1	6.22, 2.40	
13	48.1	-	-	-	-		**13**	47.9	-	-	-	-	
14	84.7	-	-	-	-		**14**	84.7	-	-	-	-	
15	α31.5	1.80					**15**	α					
	β	1.97						β					
16	α21.8	1.795					**16**	α 22.7					
	β	2.03						β	2.08				
17	α50.6	2.47	t; 9.0	48.1, 21.8, 18.3	2.72, 1.69-1.79, 1.20		**17**	α 50.7	2.385	t; 8.9	47.8, 22.7, 18.1	2.08	
18	β18.3	0.90	s	84.7, 50.6, 48.1, 39.2	2.42, 2.01, 1.96, 1.20		**18**	β 18.1	0.825	s	84.4, 50.5, 47.9, 39.1	-	
19	β31.7	1.105	s	136.3, 51.7, 41.0, 37.6	6.29, 2.45, 2.08, 1.71		**19**	β 30.7	1.174	s	136.2, 52.2, 40.2, 38.2	-	
20	77.7	-	-	-	-		**20**	85.5	-	-	-	-	
21	20.9	1.20	s	77.7, 77.3, 50.6	3.38, 2.72, 2.47, 2.42, 1.37, 0.90		**21**	22.3	1.167	s	85.5, 82.9, 50.6	-	
22	77.3	3.38	dd; 10.7, 1.7	77.7, 36.4	2.07, 2.01, 1.78, 1.72		**22**	83.0,	3.71	d; 9.1	-	1.50, 1.45	
23	*a* 31.0	1.37	m				**23**	*a* 27.9	1.45		-	3.71, 1.28	
	b	1.71		36.4	3.38, 2.47			*b*	1.50				
24	*a* 36.4	2.08					**24**	*a* 37.6	1.28				
	b	2.29	ddd; 14.6, 9.7, 4.8	-	1.38			*b*					
25	147.0	-	-	-	-		**25**	29.4	1.60				
26	110.9	4.73	s	36.4, 22.8	-		**26**	22.95	0.92	s	37.6, 29.4, 23.0	-	
		4.73	s	36.4, 22.8	-								
27	22.8	1.75	s	147.0, 110.9, 36.4	4.73		**27**	23.0	0.925	s	37.6, 29.4, 23.0	-	
							28	108.1	-	-	-	-	
							29	27.1	1.31	s	108.1, 29.5, 30.7	-	
							30	29.4	1.39	s	108.1, 27.1	-	
							31	109.6	-	-	-	-	
							32	α 26.7	1.30	s	109.6, 28.8	-	
							33	β 28.9	1.49	s	109.6, 26.7	-	

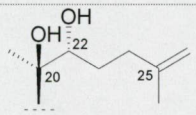
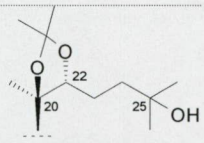
3.5.4. Structure of the ecdysteroids

Tables 12 and **13** indicate the structures of all the prepared ecdysteroids. The IUPAC names of the new compounds are given in **Table 14**. New natural compounds are denoted (*), and semisynthesized compounds are denoted (**).

Table 12. Structures of classical 7-en-6-one ecdysteroids



Ecdysteroid	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	R^{10}	R^{11}	R^{12}
Ajugasterone C	OH	βOH	H	OH	αOH	OH	H	αOH	H	H	H	H
Ajugasterone C 2,3;20,22-diacetonide (*)			H	OH	αOH							
Ajugasterone C 20,22-acetonide	OH	βOH	H	OH	αOH							
Ajugasterone D	OH	βOH	H	H	αOH							
22-Deoxy-20,21-dihydroxyecdysone	OH	βOH	H	H	αOH	OH	O H	H	H	H	OH	H
22-Deoxy-20E	OH	βOH	H	H	αOH	OH	H	H	H	H	OH	H
20,26-Dihydroxyecdysone	OH	βOH	H	H	αOH	OH	H	αOH	H	H	OH	OH
E	OH	βOH	H	H	αOH	H	H	αOH	H	H	OH	H
3-Epi-20E	OH	αOH	H	H	αOH	OH	H	αOH	H	H	OH	H
14-Epi-20E(*)	OH	βOH	H	H	βOH	OH	H	αOH	H	H	OH	H
22-Epi-20E	OH	βOH	H	H	αOH	OH	H	βOH	H	H	OH	H
5α-20E	OH	βOH	αH	H	αOH	OH	H	αOH	H	H	OH	H
20E	OH	βOH	H	H	αOH	OH	H	αOH	H	H	OH	H
20E 2,3;20,22-diacetonide			H	H	αOH							
20E 20,22-monoacetonide	OH	βOH	H	H	αOH							
11α-Hydroxypoststerone(*)	OH	βOH	H	OH	αOH							
Isovitexirone	OH	βOH	H	OH	αOH							
Makisterone A	OH	βOH	H	H	αOH	OH	H	αOH	H	CH_3	OH	H
Makisterone C	OH	βOH	H	H	αOH	OH	H	αOH	H	C_2H_5	OH	H
Polypodine B	OH	βOH	OH	H	αOH	OH	H	αOH	H	H	OH	H
Pterosterone	OH	βOH	H	H	αOH	OH	H	αOH	H	αOH	H	H
Turkesterone	OH	βOH	OH	OH	αOH	OH	H	αOH	H	H	OH	H

Ecdysteroid	R^1	R^2	R^3	R^4	R^5	R^6
Dacryhainansterone	OH	OH	H	OH	OH	H
Herkesterone(*)	OH	OH	OH	OH	OH	OH
25-Hydroxydacryhainansterone(*)(**)	OH	OH	H	OH	OH	OH
25,26-Didehydrodacryhainansterone(**)	OH	OH	H			
Dacryhainansterone 2,3;20,22-diacetonide(**)			H			
9(11)-Didehydropoststerone(**)	OH	OH	H			

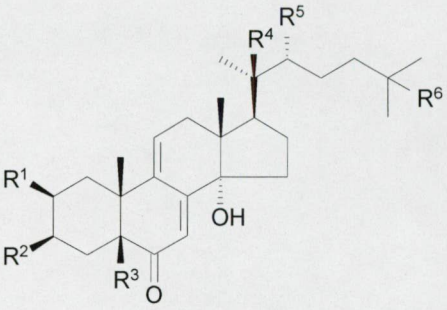


Table 13. Structures of dienone ecdysteroids

25-Hydroxydacryhainansterone was isolated and also semisynthesized from turkesterone.

Table 14. Trivial and IUPAC names of the new ecdysteroids.

	Trivial name	IUPAC name
1	Dacryhainansterone 2,3;20,22-diacetonide (**)	(20*R*,22*R*)-2β,3β,14α,20,22-pentahydroxy-5β-kolesta-7,9(11)-dien-6-one
2	24,25-Didehydrodacryhainansterone (**)	(20*R*,22*R*)-2β,3β,14α,20,22-pentahydroxy-5β-kolesta-7,9(11);25,26-trien-6-one
3	9,11-Didehydropoststerone (**)	2β,3β, 14α-trihydroxy-5β-pregna-7,9(11)-dien -6,20-dione
4	14-Epi-20E(*) (**21**)	(20*R*,22*R*)-2β,3β,14β,20,22,25-hexahydroxy-5β,14β-kolest-7-en-6-one
5	Herkesterone(*) (**18**)	(20*R*,22*R*)-2β,3β,5β,14α,20,22,25-heptahydroxy-5β-kolesta-7,9(11)-dien-6-one
6	25-Hydroxydacryhainansterone(*)(**) (**19**)	(20*R*,22*R*)-2β,3β,14α,20,22,25-hexahydroxy-5β-kolesta-7,9(11)-dien-6-one
7	11α-Hydroxypoststerone(*) (**10**)	2β,3β,11α,14α-tetrahydroxy-5β-pregn-7-ene-6,20-dione

4. Discussion

Since ecdysteroids are semipolar compounds, methanol is the most relevant solvent for their extraction. Extensive percolation with this solvent is a simple and effective method for the extraction of ecdysteroids, but such extracts contain a wide scale of contaminating compounds. Polar contaminants (such as carbohydrates, proteins, *etc.*) can be precipitated with acetone, and since the partition coefficients of ecdysteroids between hexane and 50% methanol are very low, the apolar constituents (*e.g.* most of the chlorophyll or less oxidized terpenoids) can be eliminated from the extract by using solvent-solvent partition [**132**]. Preparative-scale classical adsorption chromatography on silica is also a valuable method in

the preliminary purification. This method has a very high capacity and is cheap enough to be economic in the separation of samples in large amounts [**133-135**]. Silica can also adsorb the remaining polar contaminants strongly, while the water content of 96% ethanol can reduce the irreversible adsorption of ecdysteroids. Group separation between ecdysteroids and flavonoids could be achieved on polyamide, which adsorbs phenolic compounds more strongly, than compounds with alcoholic hydroxyl groups. Thus, the ecdysteroids were selectively eluted with water, in contrast with the flavonoids, which can be eluted at a higher solvent force (methanol) [**136**].

After preliminary purification, a multistep procedure of combined chromatographic methods was followed for the isolation of the ecdysteroids. All of these chromatographic steps (except the final HPLC) were monitored by using TLC, which is a simple and fast method, and also helped reveal 11α-hydroxyecdysteroids and dienones (see below). The TLC behaviour of the compounds predicted the numbers of free OH groups, and the choice of adequate solvent systems for their isolation was therefore easier. Three chromatographic principles were used: a normal, a reverse and a normal phase again, as shown on **Fig. 14**.

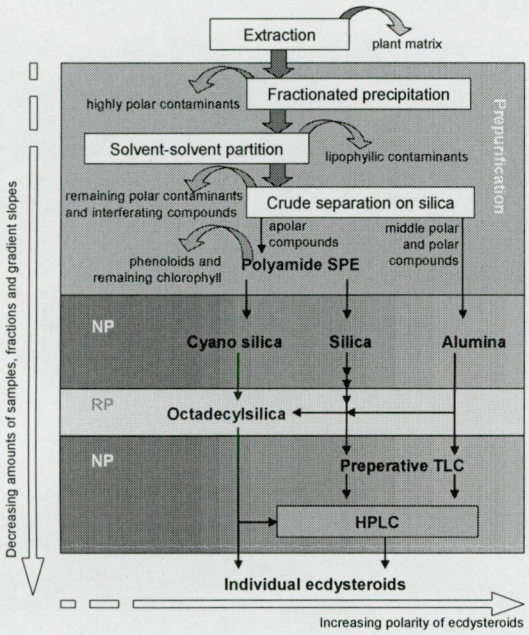


Figure 14. General strategy of ecdysteroid isolation

Summary of prepurification and three levels of combined chromatographic methods are presented.

Various polarity ranges of the SPs were used in the first, NP mode of purification, depending on the polarity of the ecdysteroids to be separated. Less polar ecdysteroids were separated on CN. This stationary phase can also be used as both NP and RP, depending on the solvent system, and if used as NP, it is the less polar. Silica and alumina were used to separate ecdysteroids with higher polarity.

The second level was RP vacuum CC [**132**]. Chemically bonded octadecylsilica was used as SP, and various rates of methanol and water as MP. Force flow was accomplished

with the use of vacuum, and the flow speed was approximately 15 ml/min. The capacity of this stationary phase is less than that of adsorption chromatography, so the columns were usually overloaded. About 10 g of sample could be separated on 100 g of stationary phase. Since separation is based on hydrophobic interactions, this method provided a notably different selectivity from that of adsorption chromatography.

The third, final level of the isolation was carried out on the NP again, using NP-HPLC. As SP, Zorbax SIL® columns were found to be the most appropriate; as MP dichloromethane-based ternary systems containing isopropanol as organic modifier and water at a maximum to 3% displayed the best selectivity **[138]**. Ecdysteroids bind NP silica quite strongly, and their peaks are susceptible to tailing. Small amounts of water can help to keep ecdysteroid peaks almost symmetric, but this decreases the reproducibility of the retention times. Water binds to the SP by the continuous use, and slowly deactivates it. The sensitivity of detection with dichloromethane-based systems may be limited by UV absorption. In cases where very small amounts were separated, cyclohexane systems (various ratios of cyclohexane - isopropanol - water) were valuable alternatives. The use of cyclohexane-based systems was also appropriate if the separation did not result in pure ecdysteroids in one step with a dichloromethane system. In such cases, with an adequate ratio of cyclohexane - isopropanol - water for the repeated HPLC, change of the selectivity was sufficient to achieve a better separation.

With combined chromatographic methods, 21 known and 4 new (11α-hydroxypoststerone, herkesterone, 25-hydroxydacryhainansterone and 14-epi-20E) natural ecdysteroids were isolated from *S. wolffii* Andrae.11α-Hydroxypoststerone is the first natural 11-hydroxylated C17-ecdysteroid. 14-epi-20E was synthesized in 2002 by Harmatha *et al.* **[139]**, and this is the first time that an ecdysteroid with a *cis* C/D-ring junction was isolated from a plant. Seven of the isolated ecdysteroids were known previously from *S. wolffii* **[88]**; the other 14 compounds are new from this plant species. Six of these 14 ecdysteroids (ajugasterone C 2,3;20,22-diacetonide, ajugasterone D, 5α-20E, 22-deoxy-20,21-dihydroxyecdysone, isovitexirone and turkesterone) were found in the *Serratula* genus for the first time **[67-78]** (see **Table 1** on page 5).

To achieve better separation of overlapping peaks, a special method of preperative HPLC was developed (see sections **2.5** and **3.3**), called peak cutting. The suggested mechanism of the process is as follows:

As the solvent component with the lowest elution force is injected (*e.g.* dichloromethane on a NP, or water on a RP), it suffers minimal retention, and passes through the column quickly. Wherever it passes, it changes the equlibria between the flowing solvent and the thin static

solvent layer around the microenvironment of the particules. This results in a significantly higher retention of any compounds which are affected. Accordingly, if the cutting solvent is eluted between two overlapping peaks, the main quantity of the later-eluting component of the sample suffers higher retention. However, in the overlapping area, some quantities of both compounds are affected together. Neither the changes in the equilibra, nor the reequilibration can be instant, so there must be two "micro-gradients" on the surface (one with a weakening and the other with a normalizing solvent force). This may explain why the effect makes a difference between the two overlapping components which are affected. The cutting is not only an illusion, but the amounts of components that remain overlapped are significantly smaller.

The manual collection of fractions is also easier, since the slope of the chromatogram between the peaks is much lower if peak cutting is used.

This method can be a quick and simple alternative to reduction of the flow rate (more time is needed) or to a change of the eluent system (more time and expensive solvents are needed).

S. wolffii was found to be particularly rich in 11α-hydroxyecdysteroids. Ajugasterone C is the main compound among them, but 11α-hydroxypoststerone, turkesterone, isovitexirone, ajugasterone C 2,3-acetonide and ajugasterone C 2,3;20,22-diacetonide were also isolated from the plant. They could be identified via their TLC behaviour after spraying of the plate with vanillin - sulfuric acid reagent. Their colour in daylight was red, which is characteristic for ecdysteroids with an 11α-OH group. This is probably associated with formation of the 7,9(11)-diene structure (together with the usually formed multiple conjugation) on the action of concentrated sulfuric acid. According to Syrov *et al.* **[30]**, 11α-hydroxyecdysteroids can have notably strong anabolic effects.

Bourne *et al.* **[140]** measured the effectivity of various ecdysteroids on the *Drosophila melanogaster* B_{11} cell line, and found that ecdysteroid 7,9(11)-dienes bind much more strongly to the ecdysteroid receptor than common ecdysteroids do. Since 11α-hydroxyecdysteroids are practically inactive in such tests, their semisynthetic transformation to dienones can produce valuable model compounds for the design of new insecticides, and/or more selective inducers for transgenic induced expression systems.

The selective dehydration of 11α-hydroxyecdysteroids has been accomplished via their adsorption onto a neutral alumina chromatographic SP at a sample/adsorbent ratio of 1:200. Dehydration proceeded in accordance with the Zajcev-orientation rule **[141]**, where alumina acts as a catalyst. The presence of an active allylic H-9 is probably the reason for the selectivity of the reaction. The HPLC-UV chromatograms revealed that ajugasterone C

2,3;20,22-diacetonide, isovitexirone, turkesterone and 11α-hydroxypoststerone were all transformed almost quantitatively (~ 90-95%, *n/n*) to the corresponding dienone.

Other reactions for the selective 9(11)-dehydration of 11α-hydroxyecdysteroids have been reported in the literature [**140,142,143**]. One of them applies NaOH [**140**] for 40 min. The authors did not report the yield of the dienone, but they performed multiple purification steps to obtain it pure. Canonica *et al.* [**142**] built protective groups onto the hydroxy-groups of muristerone A, and synthesized the 11-tosylate of the muristerone 2,3;20,22-diacetonide obtained. They then boiled the chloroform solution of the tosylate for 15 min in the presence of alumina, and kaladasterone was obtained (which is the 7,9(11)-diene corresponding to muristerone A). The authors reported the yields of the tosylate (90%) and kaladasterone (97% of the tosylate), but not the yield of muristerone A 2,3;20,22-diacetonide (it was found to be 79% by Kayser *et al.*, using a newer method [**144**]). Derivatization and the purification of the tosylate made the method complicated. Szendrei *et al.* applied alumina to obtain dacryhainansterone from ajugasterone C, with overnight mixing in methanol at room temperature; an approximate yield of 66% was achieved [**145**].

Our method is a simpler and more effective way to produce 7,9(11)-diene ecdysteroids as compared with the methods published previously in the literature.

Our isolation strategy to find possibly effective ecdysteroids also led us to the isolation of three 7,9(11)-diene ecdysteroids. Two of them (25-hydroxydacryhainansterone and herkesterone) were isolated for the first time from natural sources. Their spectroscopic behaviour helped us to identify them both by using TLC (their colour was reddish in daylight after spraying with vanillin - sulfuric acid reagent) and HPLC. On the use of dual UV detection in the ranges 240-245 nm and 298-300 nm, they could be identified by HPLC even if they coeluted with common ecdysteroids, because α,β,γ-conjugated ketones have minimal molar absorption near the wavelength of 300 nm.

However, the use of alumina SP in the first step of their isolation raised the question whether they are artefacts of 11α-hydroxyecdysteroids. To establish the presence of dacryhainansterone in the plant *S. wolffii*, three independent examinations were accomplished. A prepurified extract was used, after the steps of fractionated precipitation (and without being affected by any adsorption SP), as described in section **2.7**. By means of dual-detected RP-HPLC chromatograms, retention times and also the spectroscopic behaviour of the sample components were determined and the correspondence with dacryhainansterone was confirmed. 2D NP-TLC was also performed on the extract. In the first dimension, three-step gradient separation was accomplished, using multiple developments with increasing solvent

force. The solvent system in the second dimension contained ammonia to result in high retention of the contaminating flavonoids, due to the phenolic characteristics of these compounds. The fluorescent colour of the spot was also identical with that of dacryhainansterone after the plate was sprayed with vanillin - sulfuric acid reagent.

The presence of herkesterone and 25-hydroxydacryhainansterone in the extract was investigated by dual-detected RP-HPLC, and a peak corresponding with that of herkesterone was observed at 298 nm. Since high amounts of contaminating material were observed in the polarity range of these two compounds, the first derivative curves of the chromatograms were also calculated to make the investigation more sensitive and specific. Derivative curves supply information about the shape of the chromatogram and they can be used in the deconvolution of overlapped peaks [**146**]. Investigation of the derivative curves revealed, that the expression of a compound corresponding to herkesterone was more definite in the chromatogram of the extract if the detection wavelength was changed to 298 nm from 245 nm. The possible presence of overlapping small amounts of 25-hydroxydacryhainansterone could also be established on the basis of the corresponding local maximum and minimum in the first derivative, where the peak resolution is worse than the one for shoulder limit.

On the basis of our results, it is suggested that the isolated 7,9(11)-diene ecdysteroids are not artefacts, but natural products of *S. wolffii*. This is in accord with the results of Volodin *et al.* [**72**], who isolated dacryhainansterone from *S. coronata*, a species closely related to *S. wolffii*. However, as a consequence of the conversion of 11α-OH-ecdysteroids, it is also feasible that the isolated amounts of dacryhainansterone, herkesterone and 25-hydroxydacryhainansterone do not reflect those present originally in the plant.

5. Summary

Following our original objectives, our results may be summarized as it follows:

1. Twenty-five ecdysteroids have been isolated and characterised from *S. wolffii*
   - Eightteen of these are newly-identified ecdysteroids from this species.
   - Ten of the ecdysteroids have been isolated for the first time from the *Serratula* genus.
   - Four of the compounds have not been reported previously from any natural source.
2. Structures of the isolated ecdysteroids:
   - Six of the compounds are 11α-hydroxyecdysteroids, which may have potentially strong anabolic effects, according to the literature.
   - Three compounds have a 7,9(11)-diene structure. The known structure-activity relationships of ecdysteroids suggest that these compounds may have strong affinity for insect ecdysteroid receptors.
3. A new preparative HPLC method, peak cutting, was developed, which was successfully applied for the separation of overlapping ecdysteroids on both normal and reverse phases. The efficacy of the method was determined and statistically confirmed in the cases of 20E and 3-epi-20E on RP-HPLC.
4. A simple, economic and effective method was developed for the dehydration of 11α-hydroxyecdysteroids. Four 7,9(11)-diene ecdysteroids were obtained by using this method. Three of these compounds are new ecdysteroids. Two of them proved to be identical with specimens isolated from the plant. Each compound obtained may have noteworthy effects on insects (see above).
5. The presence of dacryhainansterone in the extract of *S. wolffii* has been confirmed by RP-HPLC and 2D TLC experiments. The natural origin of herkesterone and 25-hydroxydacryhainansterone was also suggested on the basis of dual-detected RP-HPLC.

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Annex: Papers related to the Ph.D. thesis

1.

Báthori, M., Zupkó, I., **Hunyadi, A.**, Gácsné-Baitz, E., Dinya, Z., Forgó, P.:
Monitoring the antioxidant activity of extracts originated from various *Serratula* species and isolation of flavonoids from *Serratula coronata*. *Fitoterapia* 2004, **75**: 162-167.

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Monitoring the antioxidant activity of extracts originated from various *Serratula* species and isolation of flavonoids from *Serratula coronata*

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Abstract

The antioxidant effect of aqueous methanolic herb extracts of *Serratula coronata*, *S. wolffii* and *S. tinctoria* was investigated using both enzyme-dependent and enzyme-independent systems. The extracts displayed concentration-dependent inhibition of lipid peroxidation. Flavonoids and ecdysteroids present in the extracts were evaluated as antioxidant components. The flavonoid-containing fraction of the herb extract of *S. coronata* was more effective in lipid peroxidation than the ecdysteroid-containing fraction. This paper also reports the isolation of quercetin 3-*O*-methyl ether, apigenin, luteolin, quercetin, luteolin 4′β-D-glucoside and quercetin 4′β-D-glucoside from *S. coronata*.

Keywords: *Serratula coronata*; *Serratula* spp.; Antioxidant activity; Flavonoid

1. Introduction

A great number of ecdysteroid containing whole plant preparations have been commercially available as dietary supplements, biostimulators, and OTC preparations

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[1,2]. These products are used for their anabolic, adaptogenic, hypocholesterolemic and neuroprotective effects, attributed to their ecdysteroid content.

The *Serratula* species synthesize a wide spectrum and high level of ecdysteroids and large amounts of flavonoids and are also sources of herbal remedies or food supplements [3]. The ecdysteroid composition and the 20-hydroxyecdysone content of certain *Serratula* species are described in our previous works [4,5]. However, nothing was published on the flavonoidal constituents of *Serratula* species.

Both the flavonoids and ecdysteroids have antioxidant activity and may be defined as active ingredients of *Serratula* preparation. The protective effect of flavonoids is attributed to their oxygen radical scavenging and enzyme inhibitory capabilities [6,7]. The kinetics of their chemiluminescence parameters revealed that ecdysteroids also terminate the free radical-mediated oxidation of lipids in vitro. Especially, 20-hydroxyecdysone (the main ecdysteroid) was found to be an effective lipid peroxidation (LPO) inhibitor in D-hypovitaminosis [8]. 20-Hydroxyecdysone acts by virtue of its iron-chelating or ion-reducing properties; therefore it interferes with the active iron(III) form of lipoxygenases [9].

The aim of the present study is to evaluate the antioxidant capacity of the herb extracts of *Serratula coronata*, *S. wolffii* and *S. tinctoria*, and the antioxidant effects of the flavonoid- and ecdysteroid-containing fractions of *S. coronata*. We also report here the isolation of the main flavonoids from *S. coronata*.

2. Experimental

2.1. Plant materials

The aerial parts of *S. coronata* L., *S. wolffii* Andrae and *S. tinctoria* L. (Caryophyllaceae) were collected in May 1996 in the vicinity of Budapest, Hungary. Voucher specimens (*S. coronata* S91, *S. wolffii* S94 and *S. tinctoria* S95) have been deposited in the Herbarium of the University of Horticulture, Budapest, Hungary.

2.2. Extraction for measurement of the antioxidant effect

The air-dried, crushed and powdered plant materials (5 g) were extracted with 50% MeOH in an ultrasonic bath. The extracts yielded residues of approximately 30% for *S. coronata*, 28% for *S. wolffii*, 33.6% for *S. tinctoria*.

2.3. Measurements of antioxidant activities

The enzyme-independent LPO was assayed on a standard ox-brain homogenate, and the enzyme-dependent LPO on rat liver microsomes [10,11]. In vitro experiments were conducted in duplicate and means were calculated. No error was computed; the differences between the two samples were within approximately 1%. Saturation curves were fitted to the measurement data and IC_{50} values (the correlation at which 50% of the maximum LPO inhibition is exerted) were calculated by means of the computer program GraphPad Prism 2.01.

2.4. Determination of flavonoid content

The total flavonoid content in the EtOAc-soluble fraction of each 50% MeOH extract was determined by the aluminium chloride method described in DAB 10. Each sample was analyzed three times, and a calibration graph with three points for quercetin was used. The amounts of flavonoids were expressed as quercetin (g) per 100 g of dry extract. For accuracy in the determination of the flavonoid content the *Serratula* extracts were spiked with 1 and 2 mg/ml of quercetin solution and the recovery was calculated.

2.5. Isolation of flavonoids

Dry aerial parts (118 g) of *S. coronata* were collected and extracted with 4 l of 50% MeOH using an ultrasonic bath. The extract was concentrated in vacuum to give a residue (28.18 g), which was dissolved with the aid of 140 ml of MeOH and 70 ml of acetone were added. The precipitate was filtered and washed with 3×50 ml of MeOH:acetone (1:1 v/v) solution. The filtrate and the washing solution were combined and evaporated to dryness. The residue (19.4 g) was re-dissolved in MeOH and the precipitation procedure was repeated two times [5]. The prepurified dry extract (13.9 g) was dissolved in 50 ml 50% MeOH and further purified by solvent–solvent distribution with benzene. The layer was concentrated in vacuum to give a dry residue (11.4 g) that was dissolved in MeOH and evaporated into 20 g polyamide. The polyamide with the adsorbed material was suspended in H_2O, and topped on a polyamide column (230 g, 55×8 cm). The column was eluted with H_2O (100 ml), H_2O–MeOH (8:2, 200 ml), and with MeOH (300 ml). Elution with MeOH resulted in a solution containing the crude flavonoids (4.89 g). 0.5 g of the 4.89 g flavonoid-containing fraction was dissolved in MeOH, adsorbed into 1 g polyamide and further separated on the 2nd polyamide column (10 g polyamide, 33×2 cm). EtOAc (60 ml), EtOAc:MeOH 95:5 (470 ml), 92:8 (360 ml), 85:15 (410 ml), 7:3 (70 ml), 1:1 (190 ml) were used for elution. Fractions were collected, 10 ml each.

Fractions 30–68 (0.17 g) were combined, concentrated in vacuum, dissolved in MeOH and further purified on Sephadex LH-20 column (1×53 cm). The elution was made with MeOH (1 ml fractions). Fractions 30–41 of Sephadex LH-20 column (0.06 g) were separated by PTLC on polyamide using CH_2Cl_2:MeOH 8:2. Bands with R_f 0.32 and R_f 0.16 were scraped and the flavonoids eluted with MeOH to give compound **1** (12 mg) and compound **2** (13 mg), respectively. Compound **3** (17 mg) and **4** (31 mg) were directly obtained from the 45–62 (24 mg) and 67–85 (41 mg) fractions of the Sephadex LH-20 column by crystallization.

Fractions 114–131 from the polyamide column (0.12 g) were combined, purified on Sephadex LH-20 (2×39 cm, eluted with MeOH, collected in 1 ml fractions). Chromatography on Sephadex LH-20 column resulted in compounds **5** (2 mg) and **6** (3 mg) from fractions 23–25 and 31–37, respectively.

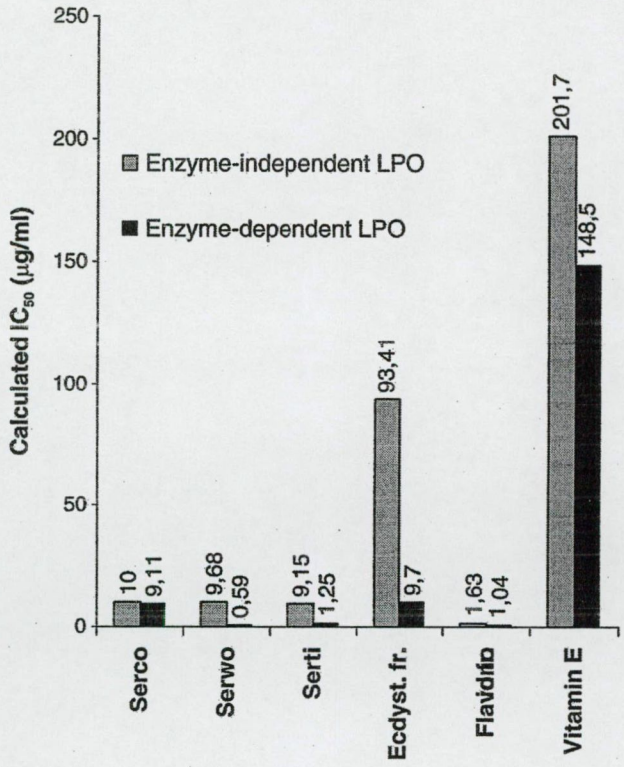

Fig. 1. Antioxidant activities of plant extracts[a], ecdysteroid and flavonoid-containing fractions of *S. coronata*, Vitamine E[b]. ([a]Serco: *S. coronata*; Serwo: *S. wolffii*; Serti: *S. tinctoria*, [b]Vitamine E=α-Tocopheryl succinate).

The structure elucidation of compounds **1–6** was performed by ^{1}H-NMR, ^{13}C-NMR, MS, IR and UV spectroscopy. The spectroscopic data of the isolated flavonoids agreed with the literature [12,13].

3. Results and discussion

The 50% aqueous methanolic extracts of the three *Serratula* species demonstrated the dose-dependent inhibition of LPO (Fig. 1). They were tested against the auto-oxidation of a standard ox-brain homogenate and against NADPH-dependent LPO in rat liver microsomes. Each of the extracts was found to be more effective in both tests than α-tocopherol acid succinate, applied as a positive control. All three extracts exhibited somewhat more potent effects in the enzyme-dependent test than in the enzyme-independent test, indicating the moderate direct enzyme-inhibitory activity of the extract, as a component of the total antioxidant effect.

The total flavonoid content of the extract of *Serratula* species was determined by means of spectrophotometry (Table 1). The quantification was controlled by the use

Table 1
Determination of the total flavonoid-content of *S. coronata*, *S. wolffii* and *S. tinctoria* by spectrophotometry

Quercetin (mg/ml)*	Total flavonoids (mg/ml)		
	S. coronata	*S. tinctoria*	*S. wolffii*
0	0.718	0.579	0.771
1	1.761	1.561	1.821
2	2.754	2.548	2.819
Average recovery (%)	101.95	98.82	102.19
Total flavonoid content (%)	0.90	0.73	0.95
R.S.D. (%)	1.99	1.56	1.48

$n=3$. R.S.D. = Relative standard deviation.
* Added as standard.

of peak addition methods. Determining recoveries from pre-analyzed solution, spiked with two different amounts of quercetin, validate the accuracy of the method. Comparison of the results of the samples and spiked samples yielded an accuracy of 98–102%.

To differentiate the LPO-inhibitory activities originating from either the ecdysteroids or the flavonoids of *S. coronata*, the extract was separated. The separation was based on the difference in the absorption/elution characteristics of ecdysteroids and flavonoids on polyamide [5]. Separation takes place after prepurification of the crude extract. The prepurification includes fractionate precipitation and solvent–solvent distribution. The prepurified extract was adsorbed on polyamide and the ecdysteroids were eluted through the successive applications of water and 20% aqueous methanol. The flavonoids remained on the polyamide, and were next eluted with 100% methanol.

The LPO-inhibitory activities of the flavonoid- and ecdysteroid-containing fractions are depicted in Fig. 1. The ecdysteroid-containing fraction displayed an approximately 10-fold higher LPO-inhibitory activity in the enzyme-dependent than in the enzyme-independent system. The calculated IC_{50} values of the flavonoid-containing fraction were approximately 60 and 10-fold lower than those of the ecdysteroids in the enzyme-independent and enzyme-dependent systems, respectively.

To establish which flavonoids are responsible for the powerful LPO-inhibitory activity, six flavonoids were isolated: quercetin3-methyl ether (**1**), apigenin (**2**), luteolin (**3**), quercetin (**4**), luteolin4′β-D-glucoside (**5**) and quercetin4′β-D-glucoside (**6**). The antioxidant effects of these flavonoids are direct consequences of their chemical structures [7]. Three of them (**1**, **3**, **4**) contain vicinal hydroxy groups, and in all of them a 2,3-double bond is conjugated to a 4-oxo group. An additional hydroxy group on heterocycle (**4** and **6**) enhances the antioxidant capacity. Among the isolated flavonoids, **4** has earlier been found to exert inhibitory effects on non-enzymatic hydroxyl radical formation, iron-ion-dependent LPO, and ascorbate-

induced non-enzymatic LPO. Compound 3 inhibits NADH-oxidase and the oxidation of succinic acid [1]. Antioxidant effects of 2 and 5 have also been described [7].

The LPO-inhibitory activities of the ecdysteroids are moderate, and more expressed in the enzyme-dependent than in enzyme-independent system. The activity may be connected to the direct enzyme-inhibitory effect rather than to the free radical-scavenging activity of any specific part of the chemical structure of the ecdysteroids. The common presence of the flavonoids and ecdysteroids in the extract may lead to a potentiating effect in the enzyme-independent LPO system, or other types of compounds may affect the activities of the extract.

The ecdysteroid content of *S. coronata* is about twice that of the flavonoid content. The majority of the LPO activity of this species is, therefore attributed to its flavonoids. The powerful antioxidant effects of the *S. coronata* extract might also make an important contribution to the uses of the products made of this species. The results of further investigations will demonstrate the contributions of the individual flavonoids to the overall effect.

Acknowledgments

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Two new ecdysteroids from *Serratula wolffii*. *J. Nat. Prod.* 2004, **67:** 1070-1072.

Two New Ecdysteroids from *Serratula wolffii*

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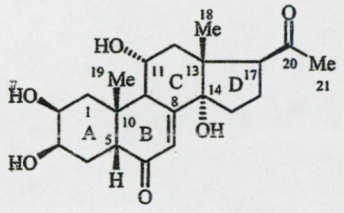
11α-Hydroxypoststerone (**1**) and herkesterone (**2**), two new natural ecdysteroids, were isolated from the herb *Serratula wolffii*. The former compound is the first 11-hydroxylated C_{21} ecdysteroid, while the latter is a new ecdysteroid with a 7,9(11)-dien-6-one chromophore. Their structures were determined using a combination of spectroscopic techniques.

Ecdysteroids are insect hormones responsible for the regulation of molting and control of embriogenesis and vitellogenesis. Phytoecdysteroids are structurally related to the main insect hormone ecdysone.[1] The diverse structural variations of phytoecdysteroids have been the basis of structure−activity experiments. These studies have proven that the ecdysteroids with a 7,9(11)-dien-6-one structural element show particularly high biological activities.[2] Ecdysteroid receptors have been engineered to be gene regulation systems and are induced by phytoecdysteroids to modulate gene expression.[3] Muristerone A[4] and ponasterone A[5] were found to be the two most active inducers, but efforts have been made to find additional inducers. The human gene therapy experiments have identified new ecdysteroids.

We report the isolation and structural elucidation of two new natural ecdysteroids from *Serratula wolffii* Andrae (Asteraceae), 11α-hydroxyposterone (**1**), and 5β,25-dihydroxydacryhainansterone (**2**). The last ecdysteroid has been given the trivial name herkesterone. Compound **1** is the first 11-hydroxylated ecdysteroid of the pregnane type that shows structural similarity to muristerone A. Herkesterone (**2**) is an ecdysteroid 7,9(11)-dien-6-one with potential insect hormone activity.

Compounds **1** and **2** were purified by solvent−solvent distribution, precipitation with acetone, and chromatographic purification from the methanolic extract of *S. wolffii*.[6] The IR spectrum of **1** showed typical absorption bands for OH and conjugated C=O, corresponding to common characteristics of ecdysteroids. The UV spectrum verified the presence of the 7-en-6-one chromophore of ecdysteroids.[7] The molecular formula, $C_{21}H_{30}O_6$, of **1** was established by the molecular ion peak, which was also consistent with the ^{1}H and ^{13}C NMR data (see Table 1). The ^{13}C NMR spectrum of **1** consist of 21 lines corresponding to three CH_3, five CH_2, seven CH, and six nonprotonated carbon atoms. Considering the chemical shifts of the CH signals, three are substituted with oxygen (HC−O) and one is =CH. Among the nonprotonated carbon atoms there are two C=O, one sp^2 =C, and three sp^3 C, where one is attached to oxygen. The HMBC correlations of the methyl hydrogens at 2.16 ppm with the C=O signal at 212.3 ppm and with the CH signal at 60.0 ppm revealed the presence



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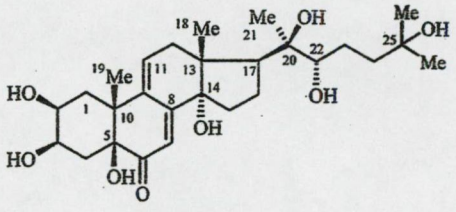

2

of an acetyl group connected to the steroid skeleton. The methyl signal at 0.61 ppm gave HMBC cross-peaks with two quaternary carbon atoms (48.6, 84.8) one methylene (42.3), and one CH (60.0). The last correlation is consistent with the acetyl group being connected to C-17 and the methyl at position 18. The HMBC connectivities of the methyl (δ_H 1.05) revealed the assignment of the C-1, C-5, C-9, and C-10 atoms. The olefinic hydrogen showed HMBC correlations to C-5, C-9, and C-14, placing the conjugated carbonyl at position 6. The ^{1}H,^{1}H-COSY correlations starting from H_2-1 (δ 2.60, 1.38) revealed the connectivities of the hydrogen atoms located in the A ring, which comprises one spin system, whereas the H-9 (δ 3.18) correlation assigned the H-11 and H_2-12 signals (ring C). The large deshielding of the H-2, H-3, H-11 and C-2, C-3, C-11 resonances are consistent with the OH substitutions. The coupling pattern of these hydrogens indicated that 2-OH and 11-OH are located in equatorial positions and 3-OH is axial. The ^{1}H and ^{13}C assignment of the atoms in ring D was supported by an HMQC-TOCSY experiment.

The NOESY correlations (Figure 1) H_3-19/H_2-1, H_3-19/H-5, H-2/H-9, and H-4/H-9 verified the *cis*-type junction of the A/B ring system. The NOESY cross-peaks of H_α-12/H_α-17, H_3-18/H_β-15, and H_3-18/H_β-16 are in accordance with the *trans*-type connection of the C/D ring system and at the same time placed the acetyl group in the β-position.

The IR spectrum of **2** showed absorption bands for OH, C=C, and C=O. The UV spectrum supported the presence of the 7,9(11)-dien-6-one structure.[7] The molecular formula of **2** was established on the basis of HRESIMS. The ^{1}H and ^{13}C NMR chemical shifts of compound **2** are summarized

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Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Spectral Data of Compounds **1** and **2** (in MeOH-d_4 and DMSO-d_6 δ in ppm)

position		1 (MeOH-d_4)			2 (MeOH-d_4)		
		^{13}C	^{1}H	mult., J (Hz)	^{13}C	^{1}H	mult., J (Hz)
1	α	39.2	2.60	dd; 13.0, 4.2	34.5	2.05	
	β		1.38	t; 12.3		2.05	
2	α	69.1	4.015	dt; 11.8, 3.8	68.6	3.83	ddd; 10.7, 5.4, 3.2
3	α	68.7	3.96	q; 2.9	70.3	3.88	q; 3.0
4	α	33.5	1.775	td; 13.6, 2.4	39.2	1.92	dd; 14.5, 2.8
	β		1.70			1.77	dd; ?, 3.4
5	β	53.0	2.345	dd; 13.1, 4.1	80.5		
6		206.6			203.1[a]		
7		123.3	5.807	d; 2.7	117.9	5.83	t; 1.2
8		164.4			156.5		
9	α	43.1	3.18	dd; 8.9, 2.7	137.9		
10		40.1			46.4		
11	β	69.4	4.08	ddd; 10.8, 8.9, 5.8	134.3	6.34	dt; 6.7, 2.0
12	α	42.3	2.406	t; 11.4	39.2	2.726	dd; 18.0, 1.8
	β		2.08	dd; 12.0, 5.8		2.436	dd; 18.2, 6.7
13		48.6			48.0		
14		84.8			84.5		
15	α	32.3	1.68		31.5	1.80	
	β		2.00			1.98	
16	α	22.4	1.90		21.8	1.79	
	β		2.245			1.99	
17	α	60.0	3.36	dd; 9.4, 8.1	50.7	2.49	t; 9.0
18	β	18.4	0.61	s	18.2	0.90	s
19	β	24.8	1.05	s	26.5	1.05	s
20		212.3			77.8		
21		31.6	2.16	s	20.9	1.208	s
22					78.6	3.35	dd; 10.3, 1.8
23	α				27.5	1.30	
	β					1.68	
24	α				42.5	1.44	ddd; 13.3, 11.6, 4.2
	β					1.81	
25					71.4		
26					29.1	1.19	s
27					29.9	1.206	s

[a] Very weak intensity in the ^{13}C spectrum.

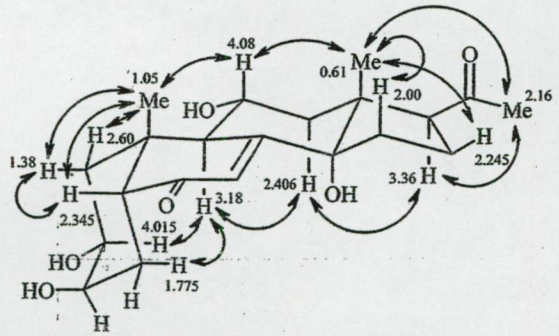

Figure 1. Stereostructure of compound **1**. The double arrows indicate the observed characteristic NOE correlations.

in Table 1. For the structure elucidation of compound **2** we utilized the same type of NMR measurements as described above. Here we discuss only the essential features of the structure elucidation. The presence of an α,β,γ,δ conjugated ketone was established on the basis of HMBC correlation of H-7 (5.83) with the quaternary =C signal at δ 137.9 and further supported by the H-11/H$_2$-12 correlations observed in the ^{1}H,^{1}H-COSY spectrum. The HMBC cross-peaks of H$_3$-19 (δ 1.05) and H-7 with the signal at δ 80.5 proved the presence of an OH substituent at C-5. The chain attached to C-17 is common in several ecdysteroids, and the observed chemical shifts for compound **2** are in accord with literature data.[7] H-2 is axial, as evident from its coupling constant of 10.7 Hz. The detected NOESY correlation between H-2 and H-11 is unique and provides straightforward evidence for the *cis*-type A/B ring junction (Figure 2). It is worth mentioning that the semiempirical calculation (HyperChem Release 7.0) showed 2.8 and 4.3 Å internuclear H-2 and H-11 distances for *cis*- and *trans*-type ring junctions, respectively. To gather further evidence for the structure and the *cis*-type A/B ring junction, a ROESY spectrum was measured in DMSO-d_6. The expected correlations of the 5-OH proton were not observed due to exchange; only the 22-OH gave COSY correlation.

Compound **1** is the first C$_{21}$ ecdysteroid with an important corticoid hydroxylation.[8] On the basis of the structure–activity studies the presence of the 7,9(11)-dien-6-one chromophore and 5β-hydroxyl and the absence of a 25-hydroxyl group usually increase the insect hormone activity

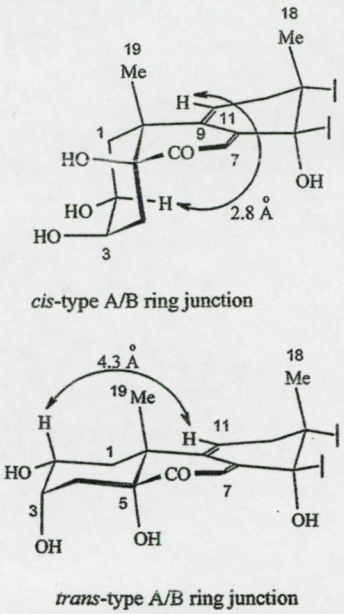

Figure 2. A/B ring junction structures of **2** and its 5α-OH isomer with the calculated (HyperChem Release 7.0) distances between H-11 and H_{ax}-2 (see double arrow).

of ecdysteroids.[2] Compound **2**, with a 7,9(11)-dien-6-one chromophore and 5β- and 25-hydroxylations, represents a new lead compound to study the common effects of these substitutions on activity.

Experimental Section

General Experimental Procedures. Melting points were measured with a Boetius apparatus (Dresden, Germany). Optical rotations were measured with a Perkin-Elmer 341 polarimeter. The UV spectra were recorded in MeOH using a Shimadzu UV 2101 PC spectrophotometer. FT-IR spectra (KBr) were recorded using a Perkin-Elmer Paragon 1000 PC FT-IR spectrophotometer. NMR spectra were recorded in MeOH-d_4 and in DMSO-d_6 in a Shigemi sample tube[9] at room temperature using a Bruker Avance DRX-500 spectrometer. Chemical shifts are given on the δ-scale and were referenced to the solvents (MeOH-d_4: δ_C = 49.1 and δ_H = 3.31; DMSO-d_6: δ_C = 39.5 and δ_H = 2.51). In the 1D measurements (^{1}H, ^{13}C, DEPT-135) 64K data points were used for the FID. The pulse programs of the 2D experiments [gs-COSY, gs-HMQC, HMQC-TOCSY (mixing time = 100 ms), gs-HMBC, NOESY (mixing time = 500 ms), ROESY (mixing time = 300 ms)] were taken from the Bruker software library, and the other parameters (pulse lengths and levels, delays, etc.) were in agreement with the parameters given in our previous work.[10] HRESIMS and FABMS were recorded on a Finnigan MAT 95SQ (Finnigan MAT, Bremen, Germany) hybrid tandem mass spectrometer. The stationary phase for the low-pressure reversed-phase column chromatography was Kovasil C18 (0.06–0.02 μm, Chemie Uetikon, Uetikon, Switzerland), and a Zorbax-SIL column (5 μm, DuPont, Paris, France) was used for HPLC.

Plant Material. The aerial parts of *Serratula wolffii* were collected in July 2001 from Herencsény, Hungary. A voucher specimen (collection number S94) was deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

Extraction and Isolation. The dried herb (2 kg) was extracted with MeOH, purified with fractionated precipitation and solvent–solvent distribution,[6] and subjected to column chromatography on silica gel. Fractions eluted before 20-hydroxyecdysone [CH_2Cl_2–MeOH, 8:2 (4.3 g)] were separated by a combination of polyamide [H_2O, H_2O–MeOH, 75:25 (3.5 g)], alumina, and silica [CH_2Cl_2–MeOH, 9:1 (0.26 g)] and low-pressure reversed-phase column chromatography. Fractions eluted with MeOH–H_2O (40:60) from the reversed-phase column (0.13 g) gave **1** (2.2 mg). The 20-hydroxyecdysone-containing fractions eluted by CH_2Cl_2–MeOH (8:2) (13.6 g) from the first silica column were purified by column chromatography on alumina. Ecdysteroids eluted after 20-hydroxyecdysone with CH_2Cl_2–MeOH (9:1) (0.67 g) were subjected to reversed-phase column chromatography. Fractions eluted by MeOH–H_2O (45:55) (3 mg) were purified by normal-phase HPLC [CH_2Cl_2-i-PrOH–H_2O (125:40:3)] to give **2** (0.7 mg).

11α-Hydroxyposterone (1): colorless crystals, mp 174–176 °C; $[\alpha]^{28}_D$ +12° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 240 (4.116) nm; IR $(KBr)_{max}$ 3320, 1718, 1653 cm^{-1}; ^{1}H and ^{13}C NMR (see Table 1); FABMS *m/z* 379 $[M + H]^+$ (100), 361 $[M + H - H_2O]^+$ (88), 343 $[M + H - 2H_2O]^+$ (40), 325 $[M + H - 3H_2O]^+$ (10), 299 (11), 282 (32), 277 (13), 249 (80), 231 (20); HRESIMS *m/z* 378.2045 (calcd for $C_{21}H_{30}O_6$, 378.2042).

Herkesterone (2): colorless crystals, mp 218 °C (dec); $[\alpha]^{28}_D$ +59° (*c* 0.1, DMSO); UV (MeOH) λ_{max} (log ε) 296 (4.02) nm; IR $(KBr)_{max}$ 3560–3200, 1650, 1602 cm^{-1}; ^{1}H and ^{13}C NMR (MeOH-d_4), see Table 1; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 5.65 (1H, s, H-7), 6.18 (1H, s br; H-11β), 2.59 (1H, d; *J* = 17.8 Hz, H-12α), 2.29 (1H, dd; *J* = 17.8, 6.6 Hz, H-12β), 1.60* (1H, H-16α), 1.89* (1H, H-16β) (*assignment can be interchanged), 2.36 (1H, t; *J* = 9.3, H-17α), 0.77 (3H, s, CH_3-18β), 0.96 (3H, s b; CH_3-19β), 1.075 (3H, s, CH_3-21), 3.13 (1H, d; *J* = 10.2, H-22), 4.42 (1OH, OH-22) COSY to H-22, 1.13 (1H, H-23a), 1.49 (1H, H-23b),1.27 (1H, td; *J* = 12.0, 4.3, H-24a), 1.65 (1H, H-24b), 1.06 (3H, s, CH_3-26), 1.09 (3H, s, CH_3-27); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 78.4 (C-5), 116.5 (C-7), 136.3 (C-9), 131.7 (C-11), 37.5 (C-12), 46.3 (C-13), 82.0 (C-14), 20.4 (C-16), 48.7 (C-17), 17.3 (C-18), 25.8 (C-19), 75.5 (C-20), 20.7 (C-21), 76.3 (C-22), 26.1 (C-23), 41.4 (C-24), 68.7 (C-25), 29.0 (C-26), 30.0 (C-27); ESIMS *m/z* 495 $[M + H]^+$ (9), 477 $[M + H - H_2O]^+$ (10), 459 (23), 443 (26), 440 (11), 422 (8), 407 (49), 394 (17), 378 (9), 361 (26), 359 (10), 323 (18), 300 (100), 199 (11); HRESIMS *m/z* 494.2885 (calcd for $C_{27}H_{42}O_8$, 494.2880).

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3.

Báthori, M., **Hunyadi, A.**, Janicsák, G., Máthé, I.:

TLC of ecdyteroids with four mobile phases and three stationary phases. *J. Plan. Chrom.* 2004, **17**: 335-341.

TLC of Ecdysteroids with Four Mobile Phases and Three Stationary Phases

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Key Words:

Planar chromatography
Mobile phases
Screening
Selectivity
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Summary

Eleven mobile phases and three stationary phases have been investigated for TLC separation of a large number of ecdysteroids. Optimization of the chromatography was based on trial and error. Use of four mobile phases on three stationary phases enabled separation of all the ecdysteroids from each other in at least one system. The TLC behavior of ecdysteroids containing different numbers of hydroxyl groups, side-chain variations, and extra double bonds, and of positional isomers and stereoisomers, is reported and interpreted. Our method is suggested for screening plant extracts for ecdysteroids.

1 Introduction

The merits of thin-layer chromatography (TLC) can be fully exploited when multicomponent mixtures are to be separated. This is required for screening ecdysteroid-containing samples of plant origin. TLC is also a preferred method for optimization of experimental conditions for isolation of ecdysteroids. The technique can be performed with numerous stationary and mobile phases simultaneously [1] and all the components of the sample are visualized on the plate. The developed separation can easily be scaled up to preparative chromatography.

Ecdysteroids are natural compounds widespread in plants and invertebrates. The structures of ecdysteroids are based on a steroid skeleton, cyclopentanoperhydrophenanthrene, usually with a complete sterine side-chain [2]. Ecdysteroid structures undergo a variety of chemical reactions at different positions on the skeleton in biosynthetic pathways which produce a wide array of ecdysteroids with similar chromatographic characteristics. Any change in the chemical structure of an ecdysteroid results in alteration of its chromatographic behavior.

Lafont et al. [3] published numerical data from parallel analysis of ecdysteroids by high-pressure liquid chromatography (HPLC) and TLC. They highlighted the selectivity of HPLC.

In this paper we report direct evidence that TLC can be used to advantage to identify a variety of ecdysteroids, even if they are very closely related. A choice of four mobile phases with three stationary phases usually enables a clear-cut decision to be made about the identity or not of any standard ecdysteroid present in a sample. A reliable decision can be made even if structural isomers are present.

2 Experimental

Plant ecdysteroids were isolated as described elsewhere [4–7]. TLC was performed on 20 cm × 20 cm glass TLC plates coated with silica gel F_{254} or with RP-18 WF_{254} and on 10 cm × 20 cm glass HPTLC plates coated with CN F_{254} (E. Merck, Darmstadt, Germany). Solvents and chemicals of the best quality available were purchased from commercial sources. The plates were developed by the ascending technique in an unsaturated glass chamber (Desaga, Heidelberg, Germany) at room temperature. The eleven mobile phases used are listed in **Table 1**.

Each ecdysteroid was dissolved in methanol to give a 0.45 mg mL^{-1} solution. Each sample (10 µL) was applied 10 mm (CN F_{254} HPTLC plates) or 15 mm (TLC plates) from the edge of the plates. Plates were developed to a distance of 90 mm (CN F_{254} HPTLC plates) or 160 mm (TLC plates). After development of the plates the ecdysteroids were detected either directly by fluorescent extinction at $\lambda = 254$ nm or by use of vanillin–sulfuric acid spray reagent. After spraying, the spots

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Table 1

The mobile phases used for normal-phase (nos 1–5, 10) and reversed-phase (nos 6–9, 11) chromatography.

No.	Components	Volume ratio
1.	Dichloromethane–ethanol (96%)	8 + 2
2.	Ethyl acetate–methanol–ammonia (25%)	85 + 15 + 5
3.	Toluene–acetone–ethanol (96%)–ammonia (25%)	100 + 140 + 32 + 9
4.	Chloroform–methanol–benzene	25 + 5 + 3
5.	Ethyl acetate–ethanol (96%)–water	16 + 2 + 1
6.	Methanol–water	6 + 4
7.	Acetonitrile–water	35 + 65
8.	Acetonitrile–water–trifluoroacetic acid	35 + 65 + 0.1
9.	Tetrahydrofuran–water	45 + 55
10.	*n*-Hexane–acetone	6 + 4
11.	Acetonitrile–water	2 + 8

were observed either in daylight or at $\lambda = 366$ nm. All samples were analyzed in triplicate.

Densitograms were recorded by use of an IBM PC-controlled Shimadzu CS-9301PC densitometer (Japan) in reflectance–absorbance mode at $\lambda = 254$ nm.

3 Results and Discussion

Ecdysteroids can be separated by TLC whether they are widely structurally different or structurally very similar. Several ecdysteroids ranging from the relatively hydrophobic 2-deoxyecdysone (containing four hydroxyl groups) to the much more hydrophilic 26-hydroxypolypodine B (containing eight hydroxyl groups) could be successfully separated in a single run (**Figure 1**). The requirement for several parallel TLC analyses is explained by the broad structural diversity of the ecdysteroids and the large number of different individual ecdysteroid components in plant extracts. An alternative would be to employ multidimensional chromatography [8]. The method of choice was parallel separations by either normal phase TLC (NP-TLC), reversed-phase TLC (RP-TLC), or cyano-phase TLC (CN-TLC) developed with several mobile phases.

3.1 Improvement of Selectivity by Using Different Stationary Phases

Some separations are preferably performed on silica as stationary phase whereas RP-TLC or CN-TLC is better for others (**Figure 2**) [9–11]. NP-TLC enables adequate separation of 2-deoxy-20-hydroxyecdysone from 24(28)-dehydromakisterone A, 5α-20-hydroxyecdysone from polypodine B, 2-deoxy-20-hydroxyecdysone from 22-deoxy-20-hydroxyecdysone, and

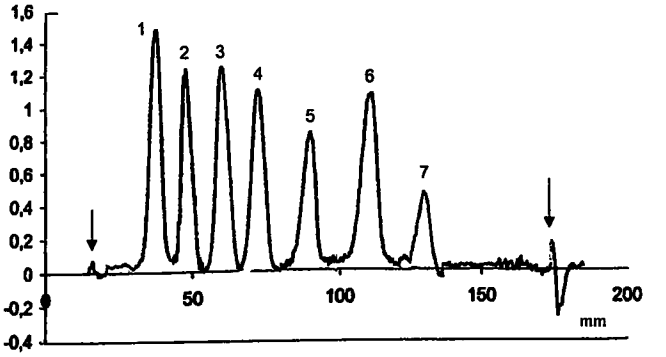

Figure 1

Separation of ecdysteroids containing from four (2-deoxyecdysone) to eight (26-hydroxypolypodine B) hydroxyl groups on TLC silica with mobile phase 5. 1, 26-hydroxypolypodine B; 2, 5α-2-deoxyintegristerone A; 3, integristerone A; 4, 22-deoxyintegristerone A; 5, 22-deoxy-20-hydroxyecdysone; 6, 2-deoxypolypodine B; 7, 2-deoxyecdysone.

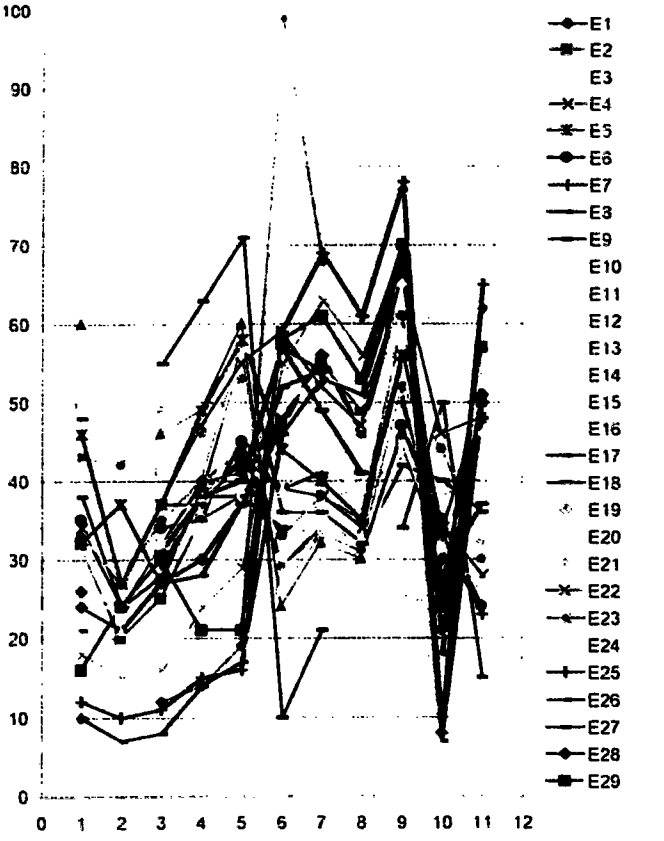

Figure 2

R_F values of some ecdysteroids separated with mobile phases 1–11. E1, 20-hydroxyecdysone; E2, polypodine B; E3, 2-deoxyintegristerone A; E4, 20-hydroxyecdysone 22-acetate; E5, 20-hydroxyecdysone 22-benzoate; E6, ajugasterone C; E7, isovitexirone; E8, 25-hydroxydacryhainansterone; E9, dacryhainansterone; E10, 5α-20-hydroxyecdysone; E11, 22-deoxy-20-hydroxyecdysone; E12, 22-deoxyintegristerone A; E13, integristerone A; E14, 24(28)-dehydromakisterone A; E15, 2-deoxy-20-hydroxyecdysone; E16, 9α,20-dihydroxyecdysone; E17, 9β,20-dihydroxyecdysone; E18, muristerone A; E19, turkesterone; E20, makisterone C; E21, 2-deoxypolypodine B; E22, rubrosterone; E23, poststerone; E24, ecdysone; E25, 26-hydroxypolypodine B; E26, 2-deoxy-20-hydroxyecdysone 22-glucoside; E27, makisterone A; E28, herkesterone; E29, 5α-2-deoxyintegristerone A.

Table 2

Effects of mobile phases on selectivity, $S^{a)}$.

Ecdysteroid	Selectivity ($\alpha - 1$) NP-TLC No. 1b)	No. 3	CN-TLC No. 10	Ecdysteroid	Selectivity ($\alpha - 1$) RP-TLC No. 6	No. 9	CN-TLC No. 11
24(28)-Dehydromakisterone A 2-Deoxy-20-hydroxyecdysone	0.2	1.04	0.93	22-Deoxy-20-hydroxyecdysone 5α-20-hydroxyecdysone	1.24	0.33	0.9
22-Deoxy-20-hydroxyecdysone 2-Deoxy-20-hydroxyecdysone	2.2	1.3	0.8	24(28)-Dehydromakisterone A 9α,20-Dihydroxyecdysone	0.43	0.51	1.09
Polypodine B 5α-20-Hydroxyecdysone	0.2	0.4	0.9	Ecdysone Isovitexirone	0.3	0.56	0.44
Makisterone C 2-Deoxypolypodine B	0.5	0.23	n.d.	Rubrosterone Poststerone	0.83	0.53	n.d.c)
	RP-TLC No. 6	No. 11	CN-TLC		NP-TLC No. 3	No. 10	CN-TLC
Ecdysone 22-Deoxyintegristerone A	0.3	2.1		22-Deoxy-20-hydroxyecdysone 5α-20-Hydroxyecdysone	0.1	1.69	
20-Hydroxyecdysone 5α-20-Hydroxyecdysone	0.1	0.33		Polypodine B 5α-20-Hydroxyecdysone	0.4	1.9	

a)$(S = [(1 - R_{F1})/R_{F1}]/[(1 - R_{F2})/R_{F2}] - 1 = \alpha - 1$ [12])
b)Mobile phase (Table 1)
c)Not determined

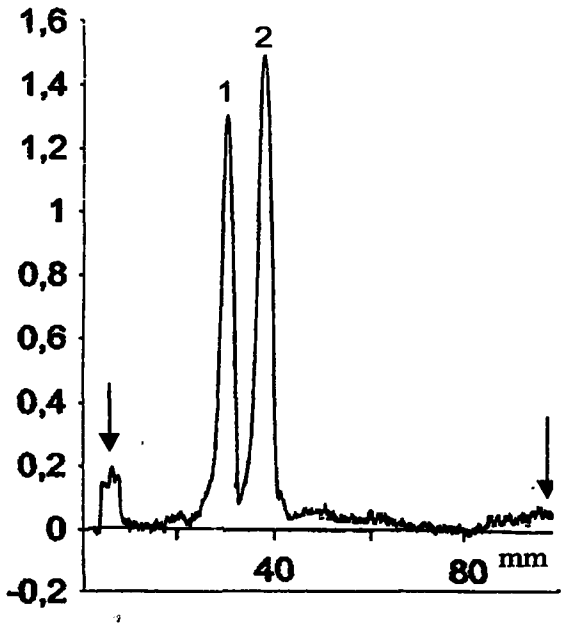

Figure 3

Separation of 20-hydroxyecdysone (1) and polypodine B (2) on HPTLC CN F_{254} plate with mobile phase 10. The spots were detected by densitometry at λ = 254 nm.

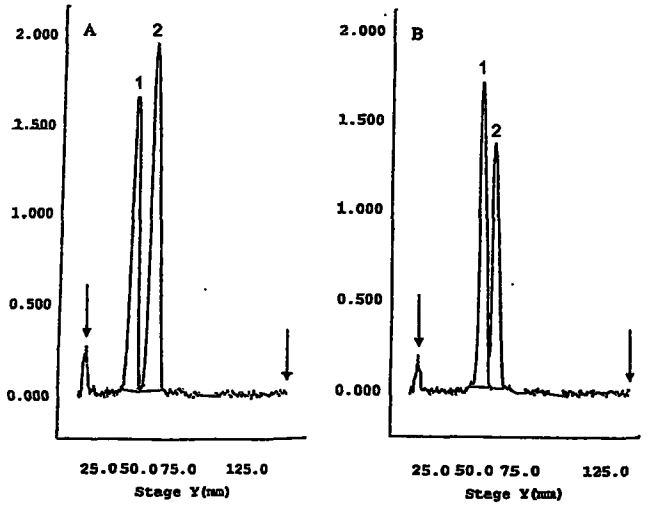

Figure 4

NP-TLC separation of 20-hydroxyecdysone (1) and polypodine B (2) on silica gel with mobile phases 1 (A) and 4 (B). The spots were detected by densitometry at λ = 254 nm.

makisterone C from 2-deoxypolypodine B. These pairs are very different structurally and, accordingly, their co-migration in RP-TLC cannot be explained simply. NP-mode CN-TLC enables better separation than RP mode for these pairs of compounds except for one pair, 5α-20-hydroxyecdysone and polypodine B, for which RP-mode CN-TLC also enables appropriate separation. **Table 2** shows the selectivity of a variety of combinations of stationary and mobile phases for these pairs of compounds.

A long-standing problem in ecdysteroid chromatography has been the separation of polypodine B and 20-hydroxyecdysone by reversed-phase chromatography, TLC and HPLC alike [13]. The mobile phase in RP mode is poorly selective for separating 5β-OH ecdysteroids from their 5β-H analogs such as for poly-

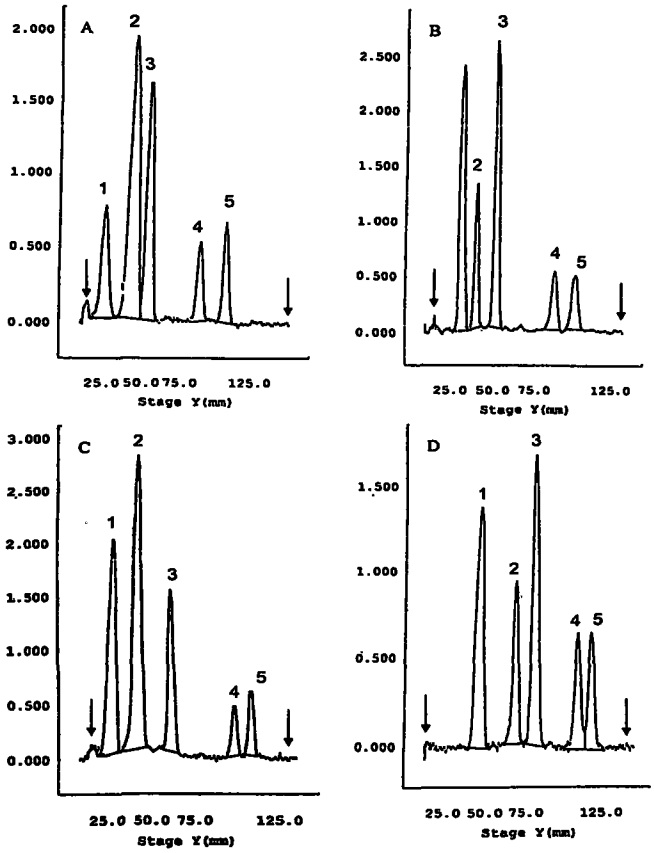

Figure 5

Densitograms, on silica gel with mobile phases 1 (A), 2 (B), 3 (C), and 5 (D), of several ecdysteroids containing different numbers of hydroxyl groups. The spots were detected by densitometry at λ = 254 nm. 1, 26-hydroxypolypodine B; 2, integristerone A; 3, 20-hydroxyecdysone; 4, 2-deoxy-20-hydroxyecdysone; 5, 2-deoxyecdysone.

podine B and 20-hydroxyecdysone. Separation of polypodine B and 20-hydroxyecdysone was achieved successfully by CN-TLC in normal-phase mode (**Figure 3**). NP-TLC on silica with some mobile phases can also be used to separate polypodine B and 20-hydroxyecdysone (**Figure 4**). Similar separation of 25-hydroxydacryhainansterone and herkesterone was achieved by use of CN-TLC (in both NP-TLC and RP-TLC modes) or silica (Figure 2). The difference between the structures of these compounds is also one hydroxyl group at C-5.

The pairs 5α-20-hydroxyecdysone and 22-deoxy-20-hydroxyecdysone, 24(28)-dehydromakisterone A and 9α,20-dihydroxyecdysone, and ecdysone and isovitexirone migrate close to each other in several NP-TLC systems (Figure 2). They can be separated by use of RP-TLC or CN-TLC (Table 2). A similar problem was observed for the separation of rubrosterone and poststerone by NP-TLC. The spots coelute even though their chemical structures are different. Because poststerone has an extra ethyne group on C-17, successful application of RP-TLC is based on the essential characteristics of the reversed-phase effect, as explained by *Horváth* and *Melander* [14].

Changes in selectivity occurred when the cyano silica stationary phase was used (Table 2). The mobility of the different ecdysteroids changed when NP-TLC was transferred from a silica to the cyano phase, and when reversed-phase chromatography was performed on cyano instead of RP18. The separation of ecdysone from 22-deoxyintegristerone A and of 5α-20-hydroxyecdysone from polypodine B changed noticeably when reversed-phase CN-TLC was used instead of RP-TLC on octadecyl silica.

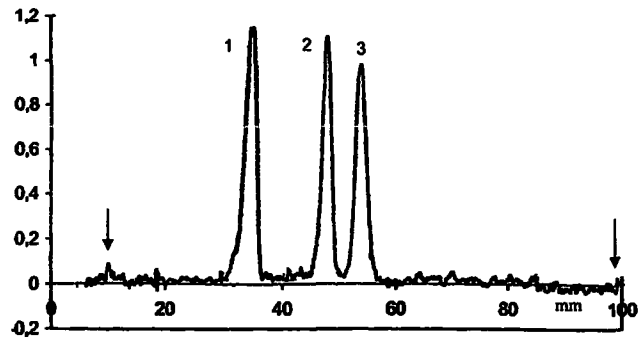

Figure 6

Densitogram of several ecdysteroids on HPTLC CN F_{254} with mobile phase 11. The spots were detected using densitometry at λ = 254 nm. 1, 20-hydroxyecdysone 22-acetate; 2, 2-deoxyintegristerone A; 3, polypodine B.

The selectivity of the normal-phase cyano system was better than that of NP-TLC for separation of some ecdysteroid pairs (Table 2), e.g. for separation of 22-deoxy-20-hydroxyecdysone from 5α-20-hydroxyecdysone and of 5α-20-hydroxyecdysone from polypodine B.

3.2 Use of Different Mobile Phases to Improve Selectivity

To establish their suitability for separation of ecdysteroids five mobile phases were tested for NP-TLC, four for RP-TLC, and two for CN-TLC. The ecdysteroids could be successfully separated by using a system of four mobile phases and three stationary phases. If two ecdysteroids co-migrate when one mobile phase is used, they can be separated by use of one of the other three mobile phases. Selection of these mobile phases was based on their excellent separating power for certain groups of ecdysteroids. Even slight differences between structures results in an appropriate difference between R_F values (Figure 2) using any of these systems. **Figure 5** shows the NP-TLC separation of several ecdysteroids using four mobile phases of different selectivity. The separated compounds differ in the number of hydroxyl groups.

The separating power of CN-TLC can be completely exploited by using two different types of mobile phase. When an apolar mobile phase is used the cyano phase behaves like a normal phase in the separation of the solutes. The interaction between the polar mobile phases and cyano stationary phase changes the order of mobility and the TLC system works as an RP-TLC system. Good separation of ecdysteroids can occasionally be achieved by use of the cyano stationary phase with mobile phase 11. Numerical data are given in Figure 2. Polypodine B, 2-deoxyintegristerone A, and 20-hydroxyecdysone-22-acetate

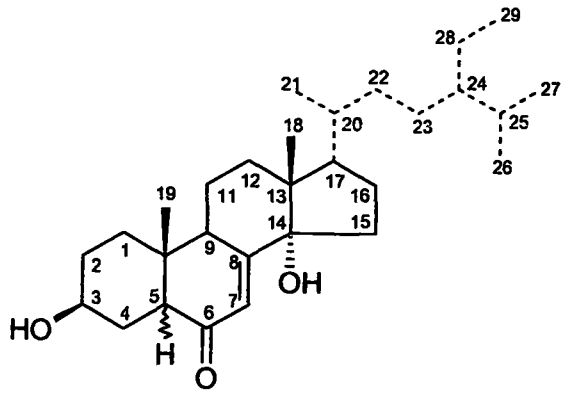

Figure 7

The general chemical structure of the ecdysteroids.

almost comigrate when the cyano phase is used with an apolar mobile phase. Separation of these compounds can be achieved by use of cyano plates in reversed-phase mode with acetonitrile–water as mobile phase (**Figure 6**).

The cyano phase offers the unique possibility of using both reversed-phase and normal-phase modes on the same TLC plate. So, 2D TLC on cyano-silica can exploit essentially different separation mechanisms in the first and the second dimensions, depending on the mobile phase used.

3.3 Effect of Hydroxyl Substitution

The chromatographic behavior of the ecdysteroids depends on the number of hydroxyl groups, on their positions, and on their orientation [15]. The positions of the hydroxyl groups strongly affect chromatographic behavior. A specific change is the consequence if a hydroxyl group in 20-hydroxyecdysone (six hydroxyl groups) is eliminated or substituted, and the change is highly dependent on the position of the alteration (**Table 3**). The same modifications (e.g. hydroxylation) at the same position cause similar effects with a variety of compound pairs. For this reason the result of hydroxylation at C-26 or C-25 was studied for the pairs polypodine B and 26-hydroxypolypodine B and dacryhainansterone and 25-hydroxydacryhainansterone (**Table 4**).

Substitution or removal of a hydroxyl group of the C-2 or C-3 diol structural moiety substantially affects the chromatographic properties and lipophilicity of ecdysteroids . A hydroxyl group at C-5 in the β orientation does not affect chromatographic mobility, or has a weak effect only. Hydroxylation at the 11, 25, and 26 positions has a similar but more pronounced effect on the chromatographic characteristics, because the change is located in a hydrophobic part of the molecules. When hydroxylation is performed at the 9α position it does not alter the lipophilicity compared with the parent 20-hydroxyecdysone. Hydroxyl groups at C-22 have an important role in determining the R_F value in RP-TLC, because of their location on the apolar side-chain. The C-25 position has a greater effect than other positions on the lipophilicity of ecdysteroids (Tables 3 and 4).

The chromatographic migration of 20-hydroxyecdysone and polypodine B are very similar in several mobile phases, as a consequence of the 5-OH group forming a hydrogen bond with the C-6 oxo group in polypodine B. This hydrogen bond counterbalances the extra (7th) hydroxyl group in polypodine B, whereas 20-hydroxyecdysone contains six hydroxyl groups. Polypodine B and 20-hydroxyecdysone are poorly separated by mobile phases 2, 3 and 5 in NP-TLC (Figure 2). Appropriate separation is achieved with mobile phase 4, and mobile phase 1 results in excellent selectivity for 20-hydroxyecdysone and polypodine B (Figure 4). The order of migration is reversed

Table 3

Effect of the position of hydroxylation on the R_F values of some ecdysteroids.

Ecdysteroid	Change relative to 20-hydroxyecdysone	R_F as a percentage relative to that of 20-hydroxyecdysone[a]								
		Normal-phase TLC					Reversed-phase TLC			
		No. 1[b]	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
20-Hydroxyecdysone	–	100	100	100	100	100	100	100	100	100
Integristerone A	C-1 + OH	75	71	59	77	78	117	113	119	106
2-Deoxy-20-hydroxyecdysone	C-2 – OH	179	200	182	148	143	62	59	66	79
Polypodine B	C-5 + OH	133	95	93	113	103	102	98	104	100
9α,20-Dihydroxyecdysone	C-9α + OH	162	n.d.	130	123	127	81	95	98	94
9β,20-Dihydroxyecdysone	C-9β + OH	116	109	118	123	102	104	96	108	99
Turkesterone	C-11 + OH	37	n.d.[c]	44	45	51	210	121	n.d.	86
Ecdysone	C-20 – OH	138	129	130	129	111	70	68	75	92
22-Deoxy-20-hydroxyecdysone	C-22 – OH	108	110	111	110	127	64	61	68	68

[a] hR_F values of 20-hydroxyecdysone are given in Table 5

[b] Mobile phase (Table 1)

[c] Not determined

Table 4

Effect of the hydroxylation at C-25 and C-26 on the R_F values of some ecdysteroids relative to those of polypodine B and dacryhainansterone.

Ecdysteroid	Change relative to parent compound (polypodine B or dacryhainansterone)	R_F as a percentage relative to that of the parent compound[a]								
		Normal-phase TLC					Reversed-phase TLC			
		No. 1[b]	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
Polypodine B		100	100	100	100	100	100	100	100	100
26-Hydroxypolypodine B	C-26 + OH	38	50	44	43	42	123	126	125	118
Dacryhainansterone		100	100	100	100	100	100	100	100	100
25-Hydroxydacryhainansterone	C-25 + OH	44	n.d.	49	44	52	n.d.	257	n.d.	200

[a] hR_F values of ecdysteroids are given in Figure 2

[b] Mobile phase (Table 1)

when mobile phases 2 and 3 are used. This is a consequence of the basic pH of these mobile phases [10]. Excellent separations of these compounds can be achieved by CN-TLC in NP mode (Figure 3), as has already been mentioned.

3.4 Changes in Selectivity for Pairs of Ecdysteroid Isomers

The order of migration of the isomer pair 5α/5β-2-deoxyintegristerone A is reversed when mobile phase 2 is used instead of mobile phases 1, 3, 4, or 5 in NP-TLC (Figure 2). Although the order of migration of this isomer pair remains the same in RP-TLC when mobile phases 6 and 9 are used, a striking difference between R_F values is observed. Adequate separation can be achieved by CN-TLC in both RP and NP modes.

Separation of the 5α/5β-20-hydroxyecdysone isomer pair is more effective in NP-TLC with mobile phases 1, 3, or 4 than in RP-TLC. NP-TLC with mobile phase 4 affords excellent separation (Figure 2). The same is true for CN-TLC, which enables better resolution of these ecdysteroid pairs in NP mode than in RP mode.

3.5 Effects of Alkyl Substitution

The usual location of alkyl substitution is on C-24. The lipophilicity of ecdysteroids is increased somewhat by alkyl substitution. The potential substituents are methyl (makisterone A), ethyl (makisterone C), and methylene (24(28)-dehydromakisterone A) groups (Figure 2).

In NP-TLC systems migration increases in the order methyl › methylene › ethyl whereas the order of increase in RP-TLC is ethyl › methylene › methyl. Mobile phase 4 results in reversal of the usual order of migration of the methyl- and methylene-substituted compounds makisterone A and 24(28)-dehydromakisterone A. The C-24-substituted ecdysteroids co-migrate in the NP mode of CN-TLC but use of the RP mode of CN-TLC results in good selectivity for these compounds.

3.6 Effect of Acetylation

Acetylation occurs in the final steps of ecdysteroid biosynthesis. Acetylated ecdysteroids are rather lipophilic and their R_F values are therefore relatively high in NP-TLC. At the same time, the spots of acetylated deoxyecdysteroids remain near the origin when RP-TLC is used. The TLC behavior of some acetylated ecdysteroids and their parent compounds are listed in **Table 5**. The position of acetylation has a substantial effect on migration – acetylation of 25-OH has the largest effect and the effect then decreases in the order 2-OH (or 3-OH) > 22-OH, as was observed in NP-TLC with mobile phases 1 and 2.

3.7 Effect of the Double Bond

The general chemical structure of ecdysteroids contains a double bond ($\Delta^{7(8)}$) (**Figure 7**). Formation of another double bond changes the R_F value. Figure 2 shows minor differences between the migration of 20-hydroxyecdysone and 25-hydroxydacryhainansterone ($\Delta^{9(11)}$), and between ajugasterone C and isovitexirone ($\Delta^{25,(26)}$), for use of several mobile phases.

3.8 Effect of Side-Chain Cleavage

Minor changes are caused by side-chain cleavage between C-17 and C-20 when RP-TLC is used (Figure 2), except for mobile phase 6. The hydrophobic side-chain contains three hydroxyl groups which are removed at the same time. For this reason rubrosterone migrates similarly to 20-hydroxyecdysone in several RP mobile phases. In NP-TLC rubrosterone has a higher R_F than 20-hydroxyecdysone. The probable reason is that side-chain cleavage has only a minor effect on retention whereas loss of hydroxyl groups is more important. Another site of cleavage of the side-chain is between C-20 and C-22. This causes a substantial change in retention when either NP-TLC or RP-TLC is used (Figure 2).

3.9 Detection of Ecdysteroids – A Virtual Separation

Because ecdysteroids absorb intensely in the ultraviolet region at 240–260 nm, a general method of detection is to view the dark spots under $\lambda = 254$ nm light on TLC plates containing a fluorescent indicator (fluorescence extinction) [16]. The ecdysteroids also give a color reaction if the plates are sprayed with vanillin–sulfuric acid reagent; the spots obtained are observed in daylight and at $\lambda = 363$ nm. The color of the ecdysteroid spots

Table 5

***hR*$_F$ values (R_F × 100) of some acetylated ecdysteroids and the parent compounds.**

Ecdysteroids	NP-TLC					RP-TLC			CN-TLC		
	No. 1[a]	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	No. 10	No. 11
20-Hydroxyecdysone	24	21	27	31	37	47	56	47	66	26	48
20-Hydroxyecdysone 22-acetate	34	24	32	40	44	39	38	34	56	34	28
20-Hydroxyecdysone 2-acetate	40	48	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20-Hydroxyecdysone 3-acetate	40	48	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Viticosterone E[b]	44	50	n.d.[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Deoxyecdysone	54	49	55	51	72	18	19	21	47	46	19
2-Deoxyecdysone 22-acetate	65	58	59	58	78	07	06	06	18	63	07
2-Deoxy-20-hydroxyecdysone	43	42	49	46	53	29	33	31	52	44	33
2-Deoxy-20-hydroxyecdysone 22-acetate	59	52	56	55	62	n.d.	23	18	n.d.	49	14

[a] Mobile phase (Table 1)

[b] 20-Hydroxyecdysone 25-acetate

[c] Not determined

is sometimes specific. Therefore, a virtual separation is obtained which can be useful for identification of ecdysteroids of similar retention. The different colors of spots of 20-hydroxyecdysone 22-acetate (brown) and 22-deoxy-20-hydroxyecdysone (orange), of rubrosterone (yellow) and poststerone (brown), and of makisterone C (violet) and 2-deoxypolypodine B (green) provide a good tool for evaluation of the chromatogram.

4 Conclusion

The use of four mobile phases and three stationary phases is sufficient for screening of the ecdysteroid composition of plant extracts, because it indicates the presence of secondary metabolites. Final differentiation of the ecdysteroids might require use of the Prisma system [17] to find the optimum mobile phase. Identification of known ecdysteroids and elucidation of the structure of hitherto unknown ecdysteroids are usually performed on the basis of spectroscopic data from the isolated pure compounds, e.g. nuclear magnetic resonance spectroscopy and X-ray diffraction.

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Novel Results of Two-Dimensional Thin-Layer Chromatography

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Abstract: Various types of two-dimensional thin-layer chromatography are presented. Even using appropriately selective systems, multicomponent mixtures can result in spots around the main diagonal, but they can be spread all over the TLC plate simply by improvements in the mobile phase composition. The use of cyano-silica offers the change of normal-phase to reversed-phase separations in the first and second dimensional developments. Elution type developments can be combined with displacement chromatography; thereby, a unique possibility of different separation mechanisms can be utilized.

Keywords: 2D-TLC, Two-dimensional, Ecdysteroids, Deprenyl, Monomethyl-lysine, Displacement

INTRODUCTION

Recently, there has been increased interest in two-dimensional (2D) separations. In a computer search, the "two-dimensional" key word provided over 500 papers, and 64 of them were published in 2004.[1] There are two basic reasons why 2D-chromatography has recently commanded the interest of chromatographers working in both industry and research. In this way, the peak

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capacity of the separation of complex mixtures is multiplied.[2] In addition, the selectivity of the separation is also definitely increased if the second dimensional chromatography utilizes an essentially different separation mechanism than the first dimension. However, certain methodical problems appear in 2D-chromatography when column (or capillary) techniques are used.

The major problem is the lack of continuous transfer of the effluent from the first to second column. Both separation systems have their special separation mechanisms, so the separation on the second column can generate overlap of the peaks having been satisfactorily separated in the first column. Furthermore, both columns contribute to the increase of the peak width, and this peak-widening is additive. Both shortcomings originate from the fact that in column chromatography the expression "two-dimensional" refers to the conception of the separation mechanisms but not to the geometrical (space) orientation of the mobile phase flow.

The real solution is the use of planar chromatography, where the two-dimensional separations are often called as fingerprints. Observing a wide variety of colorful, well-separated spots on a thin-layer plate is extremely valuable. Detection may generate a third dimension for identification. However, both generation and evaluation of a proper two-dimensional planar chromatogram can hide unforeseen shortcomings. Logically, the major problems are coming from the special circumstances, which differentiate planar chromatography from the column technique, and from duplication of one-dimensional development in real two-dimensional separations.[3]

The nature of planar chromatography offers an easy solution for the gross transfer of the spots separated as outcome of the first dimensional run into the second dimensional chromatography by simply turning the plate by 90°. The problem is, however, how to "turn" the stationary phase to give essentially different separations. Whatman Inc. (Clifton, NJ) offered an evident solution by preparing TLC plates having on one side a track of reversed-phase (RP) material, while the majority of the plates consist of plain silica. The Multi-K C-S5 dual plate has a 3 cm wide C_{18} strip on the 20 × 20 cm plate, and silica covers the remaining 17 × 20 cm field.[4] Further solutions of the problems are the use of two properly selected different mobile phases. This is possible even when only plain silica or RP-silica is used. However, an easier solution is offered by either using plates with cyano-silica coating with aqueous and organic solvents, or elution and displacement chromatography in two-dimensional separations. These arrangements and their results are the subject of the present publication.

EXPERIMENTAL

Solvents and Chemicals

All solvents and chemicals were purchased from commercial sources in the highest purity grade available. L-deprenyl [selegiline hydrochloride;

(−)-N-methyl-N-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride; (R)-(−)-*N*,2-dimethyl-*N*-2-propynylphenethylamine hydrochloride] was donated by the Chinoin Pharmaceutical and Chemical Works (Budapest, Hungary; a member of the Sanofi-Sintelabo Group). ^{14}C-L-deprenyl [(−)-^{14}C-N-methyl-N-propynyl(2-phenyl-1-methyl)ethylammonium hydrochloride, 98 μCi mg^{-1}] was prepared and provided by the Institute of Isotopes Co., Ltd. (Budapest, Hungary).

Plant Extracts

The roots of *Serratula wolffii* were extracted in the usual way.[5] The extract of *Silene viridifloras* was used without any preliminary purification.[5]

Treatment of Animals

Male Wistar rats (200–250 g) were per os treated with radiolabelled L-deprenyl (5 mg/kg).[6] Urine samples were collected for 6 h.

Elution-Displacement TLC

TLC silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) were used.[7] The mobile phases were chloroform–methanol–water (7 : 5 : 1) and dichloromethane–triethanolamine (19 : 1) for elution and displacement, that is for the first and second dimensional developments, respectively.

Derivatization

Pre-TLC derivatization of formaldehyde with dimedon resulting in formaldemedon had been detailed in our previous publications.[6,8]

Detection

Ecdysteroids were detected using a "triple-detection"[5,9] involving (1) observation under ultraviolet light at 254 nm, (2) heating after use of vanillin-sulfuric acid spray reagent and observing the plates in day-light, and (3) observing the fluorescence of the ecdysteroids under the light at 366 nm (after using the spray reagent and heating). The separated bands of deprenyl metabolites were detected using an x-ray film, with an exposure time of 120 h. Details were given in an earlier paper.[6,8]

Elution 2-D TLC

In both dimensions in normal-phase elution TLC silica gel 60 F_{254} plates from Merck KGaA were used.[7]

In normal phase vs. reversed-phase elution, the stationary phase was LiChrospher® CN (Merck), 10 μm spherical silica particles with γ-cyanopropyl function, having 10 nm pore size, 1.25 mL/g pore volume, and 350 m^2/g specific surface area; the carbon coverage was 6.6%.[7]

The following mobile phases were used:

Mobile phase No. 1.: acetone–ethanol (96%)–ammonia (25%) (140 : 3 : 9)

Mobile phase No. 2.: ethyl acetate–ethanol (96%)–water (16 : 2 : 1)

Mobile phase No. 3.: toluene–acetone–ethanol (96%)–ammonia (25%) (100 : 140 : 32 : 9)

Mobile phase No. 4.: water–acetonitrile (4 : 1)

Mobile phase No. 5.: n-hexane–acetone (3 : 2)

RESULTS

Extracts of the root of *Serratula wolffi* were subjected to the usual cleanup. The ecdysteroids (black spots) and the remaining flavonoids were analyzed using thin-layer chromatography on silica stationary phase. The mobile phases contained acetone–ethanol–ammonia and ethyl acetate–ethanol–water in the first and second dimensional runs, respectively. Figure 1 shows separation resulting in spots located mainly in the main diagonal of the TLC plate. TLC silica stationary phase was used. The same extract (side section, inner track) and an artificial mixture of the appropriate ecdysteroids standards (side section, outer track) were also separated.

Figure 2 shows separation where the spots are spread around the TLC plate. The proper mobile phase combination (Mobile phases Nos. 3 and 2) was selected here; also, the sample contained a wide spectrum of solutes, including four earlier identified ecdysteroids, and also several flavonoids.

Cyano-silica stationary phase was used to generate normal phase versus reversed phase separations on the same plate by simply changing the mobile phase composition. The ecdysteroid-containing extract was subjected to 2-D separation using a mobile phase providing straight phase separation [*n*-hexane–acetone (6 : 4)] as well as a mobile phase [water–acetonitrile (4 : 1)] adequate for reversed-phase separation on cyano-silica. The spots were detected by the so-called triple detection method that is for ecdysteroids. The 2-D TLC method resulted in adequate separation of ecdysteroids, and it can routinely be used for obtaining reliable information on the ecdysteroid spectrum of plants, and also to monitor ecdysteroid purification from plant extracts.

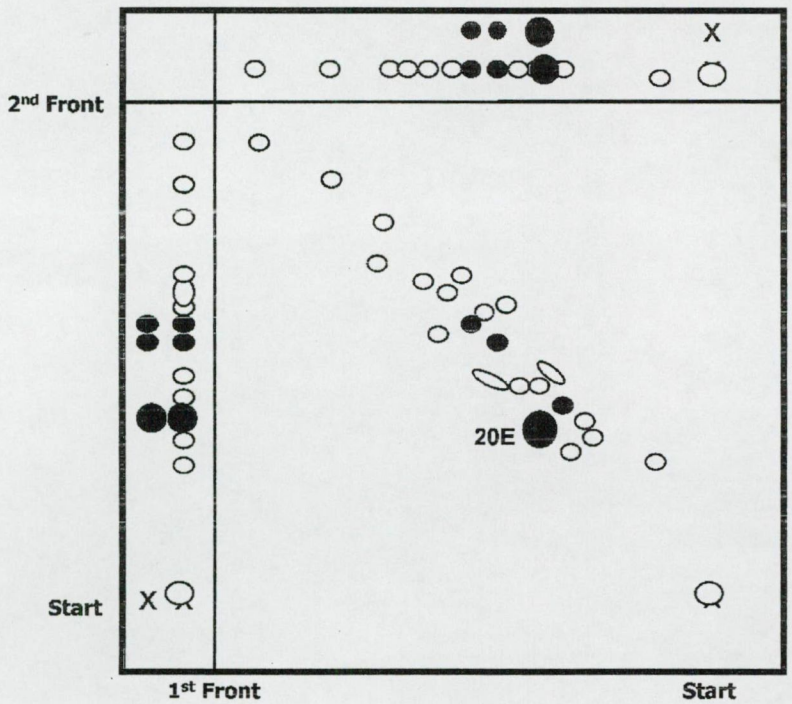

Figure 1. 2D-TLC of an extract of *Silene viridifloras*. The stationary phase was TLC silica gel F_{254}. The mobile phases were acetone–ethanol (96%)–ammonia (25%) (140 : 3 : 90) and ethyl acetate–ethanol (96%)–water (16 : 2 : 1) in the first and second dimensional runs, respectively. The spot of 20-hydroxyecdysone is marked as 20E. The same extract was loaded on both inner side tracks, and three ecdysteroids were spotted on the outer side tracks.

Figures 1, 2, and 3 are the graphical reproduction of the chromatograms. The triple-detection approach is specific for the ecdysteroids (given as dark spots). The contaminating flavonoids were detected under UV light at 254 nm, they are shown as open circles.

Elution-displacement 2D-TLC is operating under essentially different mechanisms in the first and second dimensional separations. Figure 4 presents the 2-D separation of (−)-deprenyl metabolites, including the parent drug; the sample also contained (−)-nordeprenyl, (−)-methamphetamine, (−)-amphetamine, as well as formaldemedon.

DISCUSSION

Thin-layer chromatography is carried out using a disposable stationary phase. The sample cleanup can be restricted to the removal of contaminants that

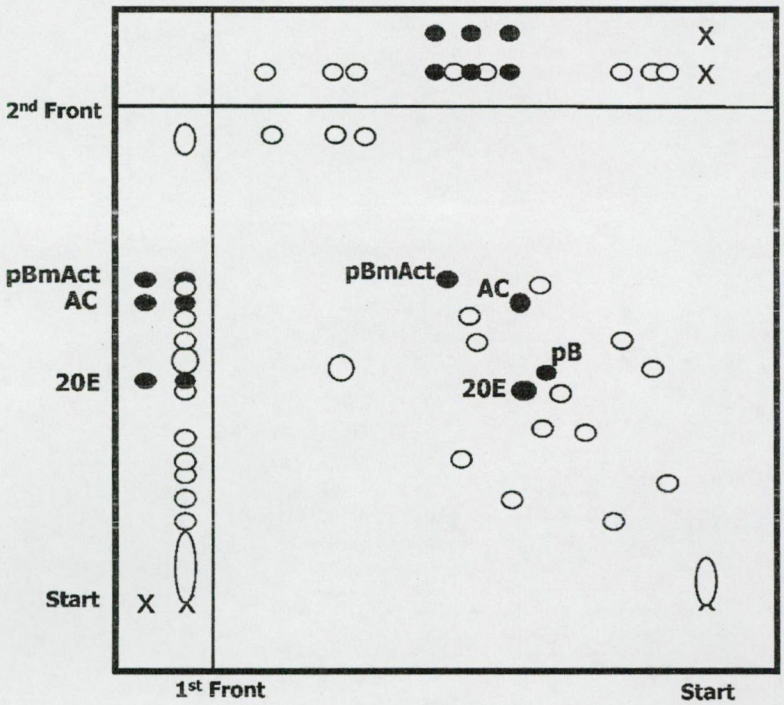

Figure 2. 2D-TLC of a root extract of *Serratula wolffii.* The stationary phase was TLC silica gel F_{254}. The mobile phases were toluene–acetone–ethanol (96%)–ammonia (25%) (100:140:32:9) and ethyl acetate–ethanol (96%)–water (16:2:1) in the first and second dimensional runs, respectively. The spots of 20-hydroxyecdysone, polypodine B, Ajugasterone C, and polypodine B monoacetonide are marked with 20E, pB, AC, and pBmAct, respectively. The same extract was loaded on both inner side tracks, and three ecdysteroids were spotted on the outer side tracks.

disturb the separation of the solutes to be determined. In situ cleanup is also possible using sesqui-dimensional development.[10] The first dimensional development serves to remove the major amount of contaminants, while the second directional development improved the separation of the important solutes.

The 2-D chromatogram is an outcome of the individual one-dimensional development. In general, the R_F value of each spot on the 2-D chromatogram has to correspond to the same solute on the side tracks. However, this is only a general rule. There are several exceptions explained by the special circumstances that differentiate the chromatographic processes in the side track from that on the 2-D TLC field. For example, 2-D-elution-displacement TLC, 2-D-reaction TLC, and sesqui-dimensional TLC belong to these exceptions.

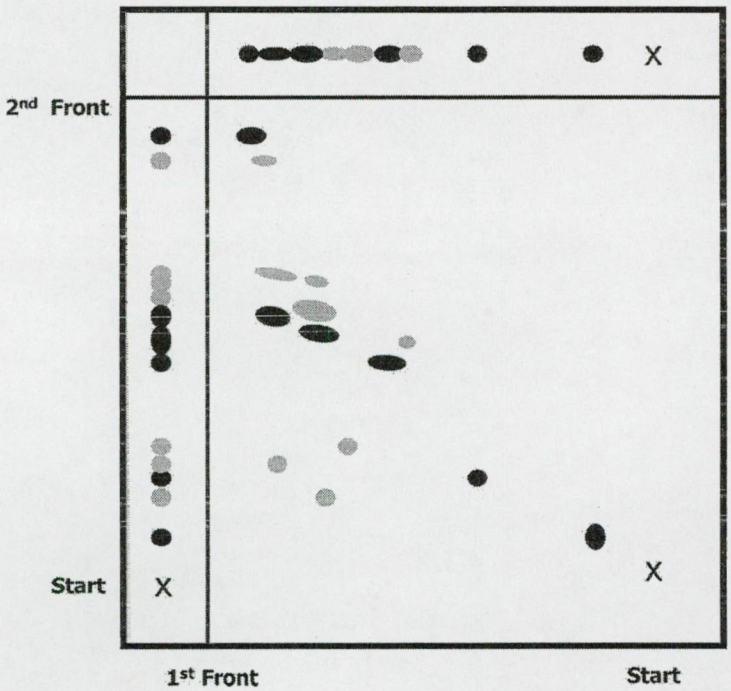

Figure 3. 2D-TLC of the extract of *Serratula wolffii*. The stationary phase was TLC cyano-silica gel F_{254}. The mobile phases were water–acetonitrile (4 : 1) and *n*-hexane–acetone (3 : 2) in the first and second dimensional runs, respectively. The spots were visualized by spraying with vanillin–sulfuric acid and observing under 365 nm UV light. The same extract was loaded on the side tracks.

The result shown in Figure 1 is an example when adequate separations were reached in both dimensional developments. However, the selectivity of the two separation systems was similar with respect to the stationary phase–mobile phase–solute combination; therefore the spots were arranged around the main diagonal of the 2-D chromatogram.

The TLC picture in Figure 2 shows a situation in which interactions among the stationary phase, mobile phases, and solutes resulted in different selectivities in the first and second dimensional developments. Therefore, the spread of the spots covers a wide portion of the 2-D chromatogram.

TLC on cyano-silica is utilizing diverging mechanisms if the first dimensional run is using a water-containing mobile phase and a water-free mobile phase is used for the second dimensional development. [*n*-Hexane–acetone (6 : 4)] generates normal-phase separation, while reversed-phase chromatography results from using water–acetonitrile (4 : 1). It is suggested that if the beneficial results are confirmed by trial-and-error, then the diverging

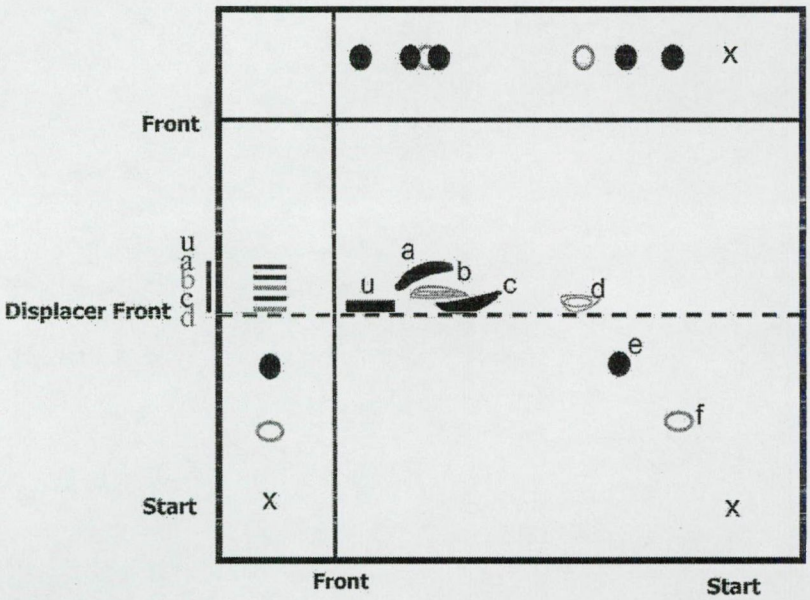

Figure 4. 2D-elution-displacement chromatography of (−)-deprenyl metabolites on TLC silica F_{254} stationary phase. The mobile phases were dichloromethane–methanol–water (7 : 5 : 1) and triethanolamine–chloroform (5 : 95) in the first and second dimensional developments, respectively. The sample components **d** (L-amphetamine) and **u** (formaldemedon = dimedon derivative of formaldehyde) were taken out from the displacement train, where the other components **a** (L-deprenyl), **b** (L-nordeprenyl), and **c** (L-methamphetamine) while subjected to a shoulder-to-shoulder displacement. Two spots, as e (para-hydroxy-L-methamphetamine) and f (para-hydroxy-L-amphetamine) were not displaced. Dark spots indicate radioactivity (and UV absorbance at 254 nm), open circles gave UV absorbance only.

separation mechanisms can even be transferred to the HPLC separation of multicomponent mixtures.

Two-dimensional displacement TLC can also be carried out using elution-type development in the first dimensional run, followed by displacement type development in the second dimension. Two distinct displacement trains have to be considered if the elution type TLC separates at least one component from the group of solutes to be displaced. The phenomenon of two discrete displacement trains is presented in Figure 4. This is the reason that only the elution development (ED) is monitored on the side track of 2D-ED-TLC.[11]

Two-dimensional reaction TLC is the case when certain solutes are chemically modified on-site between the first and second dimensional developments. Derivatization reaction does not take place unconditionally for each solute, therefore, the 2D-TLC separation cannot be derived from the one-dimensional parallel procedures at the side tracks.

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Mr. Attila Hunyadi
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Hungary

Dear Mr. Hunyadi,

I am pleased to inform you that your manuscript (Hunyadi, Gergely, Simon, Tóth, Veress and Báthori: Preparative-Scale Chromatography of Ecdysteroids of Serratula wolffii Andrea) has been accepted for publication in the Journal of Chromatographic Science.

Thank you for submitting your work to this journal.

With kind regards,

Dr. Huba Kalász

Preparative-Scale Chromatography of Ecdysteroids of *Serratula wolffii* Andrae

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Abstract

Numerous ecdysteroids have been isolated from the herb of *Serratula wolffii* Andrae, a cultivated plant. The isolation procedure included a variety of low-pressure liquid chromatography, thin-layer chromatography, gel chromatography and HPLC methods. The separation was monitored by thin-layer chromatography, and the final proof of purity was carried out by HPLC.

The isolation process involved the removal of proteins, flavonoids, chlorophylls, other sterines, etc. The purification also included the separation of the target ecdysteroids from each other. Isolation of the pure compounds required a minimum of 2 and a maximum of 8 chromatographic steps. The consecutive steps were based on the different physicochemical properties of the ecdysteroids.

Our special peak-cut method employs a flush of dichloromethane to the dichloromethane – isopropanol – water mobile phase. The flush of dichloromethane leads to an almost perfect separation of the earlier unresolved peaks.

Two ecdysteroids, 25-hydroxydacryhainansterone and 14-epi-20-hydroxyecdysone, were identified as natural products for the first time.

Structure-chiroptical relationships for some ecdysteroids are also discussed.

Introduction

The ecdysteroids are a group of steroids containing a sterane skeleton (cyclopentanoperhydrophenanthrene) with a conjugated 7-en-6-one structural element. They are highly hydroxylated compounds, with a minimum of 3 and a maximum of 8 hydroxy groups. The main structural variety is given by

- conjugation of the hydroxy groups with sulphate, acetate, cumarate, etc.,
- acetonide formation,
- glycosylation with sugars,
- the presence of an additional double bond at various positions,
- the presence of an additional oxo group.

There are three groups of steroid hormones:

1. the ecdysteroids, whose hormonal activity is restricted to arthropods, but a group of which also exert specific effects on vertebrates (for a review, see 1);

2. the vertebrate hormones, such as corticoids, androgens, gestogens, etc., which are found in all living organisms (humans, other mammalians, insects, plants, etc.); and

3. the brassinoids, which act on the growth and development of plants.

The first ecdysteroid (ecdysone, 25 mg) was isolated from about 500 kg of silkworm pupae (*Bombix mori*) in 1954 by Butenandt and Karlson (2). In 1963, Karlson et al. devised a large-scale isolation method, which resulted in 250 mg of ecdysone from 1000 kg of *Bombix mori* (3). This isolation process included several steps of extraction, solvent-solvent partition, and also chromatography, a column chromatographic separation on alumina and counter-current distribution. The final step was crystallization to produce pure ecdysone. 20-Hydroxyecdysone was isolated from silkworm and crayfish, too (4). Plants were later found to contain larger amounts of ecdysteroids, and even a wider variety of ecdysteroid structures than those in insects: currently, 260 phytoecdysteroids, but only around 100 zoo-ecdysteroids are known (5).

The substantial ecdysteroid contents of certain plants allow the use of a simple separation procedure for the main ecdysteroids. The separation pathway was optimized earlier (6-7).
The isolation of ecdysteroids may be monitored in two ways. Their ultraviolet (UV) absorbance makes it possible to employ an on-line UV detector during their HPLC separation. Moreover, their TLC separation utilizes multiple detection, one of them being based on their absorbance at 242 nm (6-7). Ecdysteroids in low concentrations have been successfully monitored by using displacement thin-layer chromatography, a method introduced by Horváth and Kalász (8-10). The enormous concentrating power of the displacement train allows the enrichment of ecdysteroids, and also their specific differentiation.

Materials and Methods

Plant material

The aerial parts of *Serratula wolffii* were collected in July 2001 from Herencsény, Hungary. A voucher specimen (collection number S94) has been deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

Thin-layer chromatography (TLC)

Normal-phase TLC (NP-TLC)

NP-TLC was performed on silica plates, 20 x 20 cm (Silica gel $60F_{254}$) (E. Merck, Darmstadt, Germany). Mixtures of

1. dichloromethane – ethanol (96%) (8:2, v/v),
2. ethyl acetate – methanol – ammonia (25%) (85:10:5, v/v/v),
3. toluene – acetone – ethanol (96%) – ammonia (25%) (100:140:32:9, v/v/v/v),
4. dichloromethane – methanol – benzene (25:5:3, v/v/v),
5. ethyl acetate – ethanol – water (16:2:1, v/v/v)

were used as mobile phases.

Reverse-phase TLC (RP-TLC)

TLC plates Whatman $KC_{18}F$, 20 x 20 cm (Whatman, Clifton, NJ, USA) were used with the following solvent systems:

6. methanol – water (4:6, v/v),
7. acetonitrile – water (35:65, v/v),
8. acetonitrile – water – trifluoroacetic acid (35:65, v/v),
9. tetrahydrofuran – water (45:55, v/v).

Cyano silica TLC (CN-TLC)

Cyano HP-TLC plates, 10 x 20 cm (CN F_{254}, 5-7 μm) (E. Merck, Darmstadt, Germany) were employed with the following solvent systems:

10. *n* – hexane – acetone (6:4, v/v),
11. acetonitrile – water (2:8, v/v).

The spots were visualized both by fluorescent quenching at 254 nm and also after spraying with vanillin-sulfuric acid and then observing in daylight or at 366 nm.

High-performance liquid chromatography (HPLC)

NP-HPLC

1. A Zorbax SIL® (DuPont, Wilmington, DE, USA), 5 μm, 250 x 4.6 mm column (for analytical purposes) and a 250 x 9.4 mm column (for preparative purposes) were used as stationary phases with the following solvent systems:

1. dichloromethane – isopropanol – water (125:50:5, v/v/v),
2. dichloromethane – isopropanol – water (125:40:3, v/v/v),
3. dichloromethane – isopropanol – water (125:30:2, v/v/v),
4. dichloromethane – isopropanol – water (125:25:2, v/v/v),
5. cyclohexane – isopropanol – water (100:40:3, v/v/v).

RP-HPLC

Stationary phases

1. A Zorbax-ODS® (DuPont, Wilmington, DE, USA), 5 μm, 250 x 4.6 mm column and
2. an Agilent Zorbax SB-C18 (Agilent Technologies Inc., Palo Alto, USA), 5 μm, 250 x 4.6 mm column were used with the following mobile phase:

1. acetonitrile – water (23:77, v/v).

The flow rate was usually 1 or 0.7 mL/min in analytical HPLC, and 2 or 4 mL/min in preparative HPLC.

Column chromatography

Different stationary phases were used, as follows:

Column 1: Silica gel 60 (E. Merck, Darmstadt, Germany) (1010 mm x 55 mm),

Column 2: Polyamide SC6 for column chromatography (Woelm, Eschwege, Germany) (300 mm x 25 mm),

Column 3: Silica gel 60 GF-254 for TLC (Reanal, Budapest, Hungary) (65 mm x 30 mm),

Column 4: Silica gel 60 GF-254 for TLC (Reanal, Budapest, Hungary) (65 mm x 34 mm),

Columns 5, 6, 8 and 9: Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) (560 mm x 20 mm),
Column 7: Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) (570 mm x 10 mm),
Column 10: Aluminium oxide Brockman II neutral (Reanal, Budapest, Hungary) (170 mm x 15 mm),
Column 11: Kovasil C18 (0.06-0.02 μm, Chemie Urticon-C-gel, Urticon, Switzerland) (450 mm x 30 mm),
Column 12: Aluminium oxide Brockman II neutral (Reanal, Budapest, Hungary) (400 mm x 45 mm),
Column 13: Kovasil C18 (0.06-0.02 μm, Chemie Urticon-C-gel, Urticon, Switzerland) (450 mm x 35 mm),
Column 14: Aluminium oxide Brockman II neutral (Reanal, Budapest, Hungary) (255 mm x 35 mm).

Extraction and prepurification of the crude extract

The dried herb (2 kg) of *Serratula wolffii* was milled and percolated with methanol (20 L) at ambient temperature. The methanolic extract was evaporated to dryness (232.9 g) and dissolved in 1250 mL of methanol, and acetone (600 mL) was added to the solution. The resulting precipitate was removed by decantation, then rinsed three times, each with 150 mL of methanol – acetone (1:1, v/v). The supernatant and the methanol – acetone solutions were combined and evaporated to dryness. The residue (166.9 g) was redissolved in methanol (700 mL), and acetone (700 mL) was added to the solution. The precipitate was washed twice with 150 mL of methanol – acetone (1:1, v/v). The supernatant and the methanol – acetone solutions were combined and evaporated to dryness. The residue (124.5 g) was dissolved in 50% aqueous methanol (500 mL) and extracted four times with *n*-hexane (4 x 1000 mL). The aqueous methanolic phase was evaporated to dryness, and the residue (108 g) was dissolved in methanol (100 mL) and adsorbed onto silica gel (200 g), using a rotatory evaporator. This was added to the top of a previously packed column of silica (1000 g, *column 1*) suspended in dichloromethane. After the column had been extensively washed and conditioned with dichloromethane (4.8 L), the ecdysteroids were eluted with dichloromethane – methanol (9:1, 85:15, 8:2, 7:3 and 1:1 v/v) (8, 7.2, 7.2, 3.2 and 4 L, respectively) and 800 mL fractions were collected. The progress of the elution was monitored by the use of NP-TLC, using solvent systems 3, 4 and 5.

Isolation of ecdysteroids

Fractions 27-30 from *column 1*, eluted with dichloromethane – methanol (9:1 and 85:15, v/v), were combined and evaporated to dryness. The dried residue (4.3 g) was dissolved in 5 mL of methanol. The solution was mixed with 9 g of polyamide and taken to dryness by rotatory evaporation. The sample was adsorbed onto polyamide and packed in a G3 glass filter (Pyrex, France) (*column 2*). Elution was carried out with water, 25% and 50% aqueous methanol and pure methanol (100 mL each), and 100 mL fractions were collected. The fractions eluted with water and 25% aqueous methanol were combined and evaporated to dryness. The dry residue (3.5 g) was dissolved in methanol (5 mL) and adsorbed onto silica (10 g), which was then added to the top of a previously packed column of silica (35 g), suspended in ethyl acetate – methanol – water

(85:10:5, v/v/v; *column 3*). Vacuum column chromatography was used with isocratic elution (ethyl acetate – methanol – water, 85:10:5, v/v/v) and 14 fractions of 25 mL were collected. Fractions 3-6 were subjected to repeated crystallization in ethyl acetate – methanol (2:1, v/v) to yield compound **1** (427 mg). The mother liquid and the dry residue of fractions 1-2 and 7-11 (2.25 g) were combined and separated by vacuum column chromatography on silica (40 g) (*column 4*). The components were eluted with a stepwise gradient of dichloromethane, dichloromethane – ethanol (98:2, 95:5 and 9:1, v/v) and methanol (250, 75, 175, 1025 and 75 mL, respectively) and 25 mL fractions were collected. Fractions 22-25 (0.43 g) eluted with dichloromethane – ethanol (9:1, v/v), were combined, and fractionated by gel chromatography on Sephadex LH_{20} (24 g), using ethyl acetate – methanol – water, 16:2:1, v/v/v, as eluent (*column 5*). 2 mL fractions were collected. Fractions 36-48 (0.24 g) were combined and separated by preparative TLC, using TLC system 2 (TLC_2). The final purification of the ecdysteroid obtained by TLC was carried out by NP-HPLC, using HPLC system 3 ($HPLC_3$), to result in compound **2** (5 mg). Fractions 35-39 from *column 4* (0.38 g) were also combined, and fractionated on Sephadex LH_{20} (*column 6*) in the same way as in the case of fractions 22-25 from *column 4*. Fractions 32-40 from *column 6* (0.158 g) were further separated by Sephadex gel chromatography (13 g) (*column 7*), using ethyl acetate – methanol (2:1, v/v) as eluent, and 1 mL fractions were collected. Fractions 11-16 (0.037 g) were finally purified by using preparative TLC_2 and $HPLC_3$ to yield compound **3** (7.2 mg). Fractions 51-60 (0.18 g) from *column 6* were fractionated by preparative TLC_2 and $HPLC_2$ to obtain compound **4** (18 mg). Fractions 40-46 (0.51 g) from *column 4* were purified by repeated use of Sephadex gel chromatography (*columns 8* and *9)* in the same way as in the case of fractions 35-39 from *column 4*. First, ethyl acetate – ethanol – water (16:2:1, v/v/v) was used and 2 mL fractions were collected. Fractions 22-25 (0.08 g) from *column 8* were further separated by Sephadex gel chromatography (*column 9)* again and the ecdysteroids were eluted with ethyl acetate – methanol (2:1, v/v); 2 mL fractions were collected. Fractions 6-10 (0.04 g) were purified by using $HPLC_3$ to yield compound **5** (13 mg). Fractions 29-38 from *column 8* (0.179 g) were recyclized onto the same Sephadex column (*column 8')* and the ecdysteroids were eluted with ethyl acetate – ethanol – water (16:2:1, v/v/v); 2 mL fractions were collected. Fractions 45-63 (0.08 g) from this column were purified by TLC_2 and $HPLC_3$ to yield compound **6** (24 mg).

Fractions 47-63 from *column 4* were combined and evaporated to dryness. The dry residue (0.63 g) was dissolved in methanol and adsorbed onto 2 g of alumina. This was added to the top of a previously packed column of alumina (18 g), suspended in chloroform (*column 10*). The ecdysteroids were eluted from the alumina with a stepwise gradient of chloroform – methanol (95:5 and 9:1, v/v; 370 and 1160 mL, respectively) and 10 mL fractions were collected. Fractions 44-138 (0.26 g), eluted with chloroform – methanol (9:1, v/v), were combined and subjected to vacuum column chromatography on octadecyl silica (180 g; *column 11*). Elution was carried out with a stepwise gradient of 30% to 60% aqueous methanol (250 mL in each step), and 50 mL fractions were collected. The ratio of methanol to water was increased by 5% in each step. Fractions 11-13, eluted with 40% aqueous methanol, gave compound **7** (2.2 mg). Fractions 23-28 (0.13 g), eluted with 50% and 55% aqueous methanol, were further purified by $NP\text{-}HPLC_2$ to obtain compound **8** (28 mg).

Fractions 31-35 from *column 1* were combined. The dry residue (13,6 g) was dissolved in 10 mL of methanol and adsorbed onto 68 g alumina, which was added to the top of 400 g of alumina previously packed into a column (*column 12*). Stepwise gradient elution was carried out with 9:1, 85:15, 8:2 and 7:3 v/v mixtures of dichloromethane – ethanol (11.1, 4.1, 1.7 and 1.8 L, respectively) and 100 mL fractions were collected. Fractions 24-30 (0.67 g), eluted with

dichloromethane –ethanol (9:1, v/v), were separated by using preparative TLC_5, which gave three well-defined zones. The ecdysteroids of these zones were further purified by $HPLC_5$ to furnish compounds **9** (3 mg) and **10** (0.7 mg) from the first, compounds **11** (1.7 mg) and **12** (2.7 mg) from the second, and compound **13** (0.7 mg) from the third zone.

Fractions 31-90 from *column 12* were subjected to repeated crystallization in ethyl acetate – methanol (2:1, v/v) to yield compound **14** (1.22 g). Fractions 91-190 from *column 12* (0.67 g) were separated, using RP vacuum column chromatography on 180 g of octadecyl silica (*column 13*) as described above, and 50 mL fractions were collected. Fractions 15-17 (3 mg), eluted with 45% aqueous methanol, were separated by NP-$HPLC_2$ to give compounds **14** (5.7 mg), **15** (0.7 mg) and **16** (1.6 mg). Fractions 21-23 (85 mg), eluted with 50% aqueous methanol, were also separated by $HPLC_2$, and 5 compounds were isolated: compounds **8** (0.6 mg) and **14** (11.7 mg) again, and **17** (8.4 mg), **18** (1.8 mg) and **19** (9.8 mg).

Fractions 41-46 (9.1 g) from *column 1*, eluted with dichloromethane – methanol (7:3 and 1:1, v/v), were adsorbed onto 45 g of alumina, which was packed on the top of a column of 270 g of alumina (*column 14*). Gradient elution was carried out with chloroform – ethanol (9:1, 8:2, 7:3, 65:35 and 6:4, v/v; 1700, 1800, 4900, 8900 and 2900 mL, respectively) and 100 mL fractions were collected. Fractions 42-60 (0.36 g), eluted with chloroform – ethanol (7:3, v/v), were combined and further fractionated, using preparative NP-TLC_5. The final purification was carried out by $HPLC_3$ to yield compound **20** (9.9 mg). Fractions 91-96 (0.11 g), eluted with chloroform – ethanol (65:35, v/v), were also purified by NP-TLC_5 and $HPLC_1$, and compound **21** (10 mg) was isolated. Fractions 97-108 (0.23 g) from *column 14*, eluted with chloroform – ethanol (65:35, v/v), were also purified by NP-TLC_5 and $HPLC_1$ to give compound **22** (11 mg).

Figure 1 shows the procedure of ecdysteroid isolation.

Physicochemical characterization and structure determination of the isolated compounds

Circular dichroism (CD) and UV spectra were determined with a Jasco J-720 Spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan).

High-resolution electrospray ionization (ESI) and fast atom bombarded mass spectra (FAB-MS) were recorded on a Finnigan MAT 95SQ (Finnigan MAT, Bremen, Germany) hybrid tandem mass spectrometer, and ESIMS-MS were determined with a Finnigan TSQ 7000 tandem mass spectrometer (Finnigan Ltd., San Jose, CA, USA).

One-dimensional (^1H, ^{13}C, DEPT-135) and two-dimensional (COSY, NOESY, HMQC, HMQC-TOCSY and HMBC) NMR spectra of ecdysteroids were taken in methanol-d_4 using a Shigemi sample tube on a Bruker Avance DRX-500 spectrometer.

Results

The ecdysteroids of the herb of *Serratula wolffii* Andrae were subjected to exhaustive extraction with methanol in a percolator at a solvent : plant ratio of 10:1. The crude extract was then subjected to prepurification, using fractionated precipitation and solvent-solvent distribution. These two consecutive purification steps removed the overwhelming majority of both the polar and the apolar contaminants. The purification was monitored by TLC.

The first chromatographic step was carried out on a silica column packed with coarse particles (0.06 – 200 μm), which resulted in fractions combined into 3 groups. The first group contained certain (about 11) ecdysteroids, but an excess of flavonoids were also eluted here as contaminants. Flavonoids were removed by using solid-phase extraction on polyamide, using a

home-made set-up. Ecdysteroids were eluted using plain water and also with 25% aqueous methanol, but flavonoids remained adsorbed on the polyamide. The adsorption-elution processes were double checked by consecutive elution with an increasing ratio of methanol, but 25% methanol always resulted in perfect elution of all the ecdysteroids present here.

Further removal of the non-ecdysteroid contaminants was achived by the repeated use of a silica gel column with medium particles (12 – 15 μm). This second silica column chromatography was carried out according to the conditions of vacuum chromatography. The stationary phase to load ratio was 13:1. In certain cases, crystallization of an ecdysteroid (pure polypodine B **1**) was observed.

The other ecdysteroids were then further separated by vacuum chromatography on silica gel again, which resulted in 4 subgroups. The first 2 subgroups were separately subjected to 3 or 4 more chromatographic purifications, i.e. to separation on a Sephadex LH-20 column or to repeated Sephadex LH-20 column chromatography and preparative TLC on silica, and NP-HPLC. Ajugasterone D **2** was separated from the other ecdysteroids on a silica column, and the further 3 steps removed only the contaminants,. The 2 ecdysteroids in the second subgroup, dacryhainansterone **3**, and pterosterone **4**, were resolved from each other by Sephadex LH-20 column chromatography. Additional Sephadex LH-20 column chromatography, TLC and HPLC removed the contaminants. A similar separation was done for the fractions combined into the third subgroup. However, column chromatography of this fraction on Sephadex LH-20 differentiated 2 ecdysteroids (20-hydroxyecdysone 20,22-monoacetonide **5**, and makisterone C **6**), and the further chromatographic steps removed the contaminants here too.

The fourth subgroup of silica fractions was subjected to alumina column chromatography to remove the non-ecdysteroid contaminants. Later, vacuum column chromatography on octadecyl silica was carried out to separate the 2 ecdysteroids present here. 11α-Hydroxy-poststerone **7**, was sufficiently pure after this separation, whereas the ajugasterone C **8** containing fractions had to be further purified by using straight-phase HPLC (silica). Eight ecdysteroids were isolated from the first fraction group from the initial silica gel column, therefore.

The second fractionation group from the initial silica gel column was subjected to separation on alumina to remove the contaminants. The column chromatography yielded 3 subgroups. The real separations of the ecdysteroids of the first subgroup were employed by preparative TLC on a silica stationary phase and NP-HPLC. In this way, 5 ecdysteroids were obtained (**9**, **10**, **11**, **12** and **13**).

The second subgroup from the alumina column chromatography contained an abundant amount of 20-hydroxyecdysone **14**, which was purified by crystallization.

The ecdysteroids of the third subgroup from the alumina column were purified by vacuum column chromatography on octadecyl silica, resulting in 2 ecdysteroid-containing fractions. The ecdysteroids of these fractions were resolved by NP-HPLC.

The detection and isolation of herkesterone **15** and 25-hydroxydacryhainansterone **16** containing 2 double bonds, was greatly facilitated by their double detection (**Figure 2**). Detection at 242 nm is characteristic of the vast majority of ecdysteroids. Ecdysteroids with the cumulated conjugation of the oxo group and the two double bonds have an additional possibility of monitoring at about 300 nm. The absorbance at 300 nm was not only specific, but also higher than that of 242 nm.

The other fraction contained 5 ecdysteroids: ajugasterone C **8**, isovitexirone **17**, 14-epi-20-hydroxyecdysone **18**, 3-epi-20-hydroxyecdysone **19** and 20-hydroxyecdysone **14** (**Figure 3**). Three of them (**8**, **17**, **18**) gave co-eluting peaks. They were separated by NP-HPLC on silica using our newly established peak-cut method.

The peak-cut method served to separate overlapping peaks. One operation improved the separation of 2 components. When preparative HPLC separation was used on a silica stationary phase with dichloromethane – isopropanol – water (125:40:3, v/v/v), the ajugasterone C **8** was eluted together with isovitexirone **17**, and isovitexirone **17** gave a peak overlapping with that of 14-epi-20-hydroxyecdysone **18** (**Figure 4/A**). First, these latter 2 ecdysteroids were separated. Insertion of a flush of dichloromethane improved the separation, and thereby the peak of 14-epi-20-hydroxyecdysone **18** separated adequately from those of isovitexirone **17** and ajugasterone C **8** (**Figure 4/B**). However, a further operational step was necessary to remove the ajugasterone C **8** from the leading part of the peak of isovitexirone **17** (**Figure 5**) using lower load.

The third fractionation group from the initial silica gel column contained 3 ecdysteroids (**20**, **21** and **22**). These were separated by using column chromatography on alumina, which was followed by their individual purification via preparative TLC and HPLC on silica. This was likewise mode of separation of further ecdysteroids with 6 and 7 hydroxy groups.
Tables 1 and **2** report the chromatographic characteristics of the isolated ecdysteroids.

Elucidation of structures of the newly isolated ecdysteroids
Comparison of the physicochemical properties and spectral data with authentic compounds allowed the identification of compounds **1-15**, **17** and **19-22**. The spectral data of **18** were compared to the synthetic 14-epi-20-hydroxyecdysone. The data were in good agreement with each other (11).
The newly isolated ecdysteroid **16** had $[\alpha]_D^{29}$ -17° (c, 0.5), and its UV spectrum in methanol gave a peak with λ_{max} (log ε) at 299 nm (3.33). The mass spectrum revealed major ions at m/z 479 $[M+H]^+$, 461.6 $[M+H-H_2O]^+$, 443 $[M+H-2H_2O]^+$, 427 $[M+H-3H_2O]^+$ and 345 $[M-C_{20}-C_{27}]^+$. The molecular weight of 478 m/z was determined by FAB-MS. This was 16 m/z higher than that of dacryhainansterone **3**, and **16** must therefore be a mono-hydroxylated derivative of dacryhainansterone **3**. The fragment ions of FAB-MS furnished evidence that **16** bears the additional OH group on the side-chain of dacryhainansterone **3**.
The ^1H and ^{13}C chemical shifts of the compound **16** is given in **Table 3**. For the signal assignment we identified at first the five methyl signals appearing as singlets in the ^1H NMR spectrum. The characteristic correlations (törlés volt) were utilised in the assignment. The identification of the geminal Me-26 and Me-27 groups are straightforward owing to their mutual HMBC correlation, whereas Me-21 correlates with two OH substituted carbon atoms exhibiting strong deshielding (~77-78 ppm). The differentiation between H_3-19 and H_3-18 atoms of the angular methyl groups was achieved considering the coupling of the latter with C-17, which also coupled to H_3-21. In accordance with a 6-oxo-$\Delta^{7,8}$-moiety, H-7 olefinic hydrogen (5.75 ppm) correlates with C-5, C-9 and C-14 carbon atoms. The high ^{13}C chemical shift value of C-10 (136.3 ppm) and the correlations of the other olefinic hydrogen (6.29 ppm) to C-8, C-10 and C-13 in HMBC spectrum of compound **16** justify the presence of a $\Delta^{9,11}$ double bond. The hydrogen atoms of ring-A form a common spin-system which was analysed by $^1H,^1H$-COSY and HMQC-TOCSY experiments. The assignments of rings-C and -D, as well as the side-chain attached to C-17 were obtained in an analogous way.
The H-19/ H_2-1 and H-19/H-5 correlations in NOESY spectrum of **16** prove (nem proves) *cis* type junction of rings-A/B. The H_β-12/ H-19, H_β-12/ H-21, H_α-12/ H-17 cross-peaks verify the *trans* type junction of rings-C/D.

Chiroptical analysis of ecdysteroids

In ecdysteroids with 7–en–6–one structure, the conjugated enone grouping is inherently chiral and the torsion angle of this grouping is of great importance for the sign of CD bands (12).

The main ecdysteroid, the 20 – hydroxyecdysone **14** and other ecdysteroids **1, 7-8, 10-11, 18, 21**, etc. are bearing a positive Cotton effect in the n → ∏* electronic transition range at 328 – 333 nm wavelength. The ∏ → ∏* electron transition explains that a sharp negative Cotton effect curve can be seen at the majority of ecdysteroid **1, 7-8, 10-11, 14,18, 21** at 247 – 257 nm wavelength.

The deviating CD curves of ecdysteroids will be interpreted in the followings.

The dacryhainansterone **3** with 7,9(11)-dien–6–one structure causes a batochromic shift of the Cotton effect curve (λ_{max}: 345 nm). The second double bound in 9 position causes the extension of the conjugation and the maximum of the negative Cotton effect curve (due to ∏ →∏* transmission) will be shifted to the longer wavelengths with 46 nm. The change of the UV spectrum is of the same extent.

The ecdysteroid with pregnane skeleton, the 11α-hydroxy-poststerone **7**, which doesn't contain a long side chain has a chirally perturbed saturated ketone chromofore in the 20 position. Its positive Cotton effect at 289 nm can be easily separated from the CD signal having a maximum at 332 nm due to the unsaturated ketone chromofore n → ∏* transition.

The maximum of the Cotton effect in the ∏ → ∏* electron transmission will have hypsochromic shift with 6-7 nm at the 5α- ecdysteroids (compound **11**). The intensity of this band increases in a great extent. The intensity of short wavelenght band at 221 nm decreases comparing to the 5β- ecdysteroids.

Characteristic is the CD spectrum of the 14–epi ecdysteroid **18.** This ecdysteroid bears a β–hydroxy substituent in 14 position. The intensity of the R – band decreases markedly. The Cotton effect, due to ∏ → ∏* electron transition will have a hypsochromic shift and the band at short wavelengths can not be measured.

The chiroptical data of the isolated ecdysteroids are given in **Table 4.**

Discussion

Serratula wolffi Andrae belongs in the Asteraceae family, a family with numerous species rich in ecdysteroids (13-20). Accordingly, determination of the ecdysteroid profile of *Serratula wolffii* Andrae appeared a worthwhile goal. There are two distinct ways to screen the profile. One is direct analysis of the extract, while the other is to isolate the ecdysteroids giving the profile (21-24). Direct HPLC was not possible because of the lack of adequately specific determination for each of the individual 260 ecdysteroids. The effects of saponoids, flavonoids and other phenoloids can disturb the analytical methods, when TLC, HPLC or even HPLC-MS may be employed. Isolation of the various ecdysteroids is a reliable method of establishing the ecdysteroid spectrum (5). Moreover, the pure ecdysteroids isolated can be checked as concerns their physicochemical characteristics.

Twenty-two ecdysteroids have been isolated from *Serratula wolffii* Andrae. 20-Hydroxyecdysone **14**, the main ecdysteroid, was purified in the simplest way: two column chromatography yielded fractions pure enough for its crystallization. Three chromatographic steps were needed to isolate polypodine B **1**, the most frequently biosynthesized phytoecdysteroid after 20-hydroxyecdysone **14**. The isolation procedure from 2 kg of dried herb of *Serratula*

wolffii resulted in 1,2 g of 20-hydroxyecdysone **14** and 0.4 g of polypodine B **1** in pure form. The overall recovery of these purifications was as high as 30 to 40%.

The other ecdysteroids were purified by an optimum combination of separation methods. Repeated absorption chromatography using a column and/or a planar technique and gel chromatography on Sephadex LH-20 were carried out, which was followed by NP-HPLC. The gel chromatography on Sephadex LH-20 may be substituted by vacuum RP column chromatography. The ecdysteroids could be separated by gel chromatography on the basis of the very small differences in their molecular masses (2-12 m/z), that and hence in their adsorption. Moreover, their solubilities made it possible to dissolve them in solutions concentrated enough for direct loading onto a chromatographic system, while the low-pressure RP column chromatography was operated by drying the sample on octadecyl silica, and transfering it to the top of the column.

The final purification generally required straight-phase HPLC. In certain cases, it was the 5th step of the use of silica, but it was the very first use of fine-particle silica packed into an HPLC column, the separation being carried out under high pressure. The exception was 11-hydroxypoststerone **7**, which was sufficiently pure without this final straight-phase HPLC. Naturally, altogether 6 isolation/chromatographic steps resulted in the purification of 11-hydroxy-poststerone **7**.

Particular procedure was employed to improve the separation of isovitexirone **17** from 14-epi-20-hydroxyecdysone **18** (**Figure 4**) and from ajugasterone C **8** (**Figure 5**). The method is called peak-cut by solvent. The HPLC separation was done by using silica gel as stationary phase and dichloromethane – isopropanol – water as mobile phase. The method includes isocratic elution with a flush of dichloromethane.

We give here the explanation of this method for the separation of isovitexirone **17** and 14-epi-20-hydroxyecdysone **18**. For preparative-scale separation (given in **Figure 4**) 100 μL of dichloromethane was injected after the load. Preliminary calculation led to the result that the dichloromethane flush reached the moving zones of isovitexirone **17** and 14-epi-20-hydroxyecdysone **18** (**Figure 4/A**) just before the end of the HPLC column, and this dichloromethane flush was inserted between the peaks of these two ecdysteroids. For preparative separation, the load was 4 μg /μL, which means an gross load of 400 μg.

The tentative mechanism of this peak-cut is as follows. Dichloromethane is the least polar component of the mobile phase. The conditions allow its migration with minimal retention. When the dichloromethane reaches the minimum between the isovitexirone **17** and 14-epi-20-hydroxyecdysone **18** (**Figure 4/A**), the isovitexirone **17** is before the zone of dichloromethane, and therefore isovitexirone **17** can be eluted without any delay. However, dichloromethane overruns the zone of 14-epi-20-hydroxyecdysone **18** , and a micro-portion of the mobile phase will be richer in dichloromethane. As the dichloromethane-rich mobile phase induces a slower retention, the zone of 14-epi-20-hydroxyecdysone **18** is subjected to a delay (**Figure 4/B**). However, it is only a very small portion of the mobile phase which generates an increased retention time and it does not influence the gross shape of the peak of 14-epi-20-hydroxyecdysone **18**. As we intended the separation of this ecdysteroid pair (isovitexirone **17** and 14-epi-20-hydroxyecdysone **18**) together with the parallel separation of two further ecdysteroids, 3-epi-20-hydroxyecdysone **19** and 20-hydroxyecdysone **14** (**Figure 3**), change of the isocratic mobile phase was not preferable.

If the precise mechanism is considered, the whole procedure means a real isocratic elution with a micro flip-flop change in the mobile phase.

Only 6 ecdysteroids with 2 double bonds (conjugated to each other, and also to the oxo group) were known earlier (5). We recently discovered 2 natural ecdysteroids containing 7,9(11)-dien-6-one structural elements, which were named herkesterone **15**, (19) and 25-hydroxydacryhainansterone **16**. The conjugation of 2 double bonds and an oxo group drastically changes their UV spectrum (25). The maximum of their UV absorbance is shifted to about 300 nm (298.6 nm for herkesterone **15** and 399.2 nm for 25-hydroxydacryhainansterone **16**). Monitoring the chromatogram at 300 nm permits their specific detection, and a virtual separation of herkesterone **15** and 25-hydroxydacryhainansterone **16** was achived when these ecdysteroids were traced in multicomponent samples. Utilization of the specific monitoring of herkesterone **15** and 25-hydroxydacryhainansterone **16** (and other, hitherto unknown ecdysteroids with 2 double bonds) opens up new possibilities for their detection in other plants.

Some recent publications have reported on the ecdysteroid spectra of certain *Serratula* species (13-20). Such reports are extremely useful for establishing plants as raw materials for medicinal preparations. Moreover, these accounts permit the chemo-taxonomical systematization of the plants. The analysis of the ecdysteroid spectrum *of Serratula wolffii* Andrae was discussed by Miladera et al. (18) and Hunyadi et al. (20). Our report essentially supplements their findings. Two ecdysteroids, the 25-hydroxydacryhainansterone **16** and the 14-epi-20-hydroxyecdysone **18** are newly discovered natural products. At the same time, they have been prepared by semisynthetic procedures (11, 25). Moreover, the ecdysteroid profile of a species depends on various factors, such as the location of the plant, the soil, the climate, the time of harvesting, etc.; thus, it depends on the surrounding ecosystem as well as on the genetic markers of the individual plant (26).

The CD spectra of ecdysteroids furnish information of the structures of these compounds, but only a few data are available in the literature on this field (5).

We have determined the CD and UV spectra of 9 compounds. On the basis of the molecular ellipticity and molar absorption values, we could establish some structure – chiroptical relationships. These results are mainly based on the evaluation of changes in the CD spectrum of the 7-en-6-one chromophore group depending on its stereochemical environment. In some cases the electron excitation spectra afford valuable information, and promote an understanding of the relationship between structure and CD spectrum.

Conclusions

Serratula wolffii Andrae, a cultivated plant, is a rich source of ecdysteroids. These ecdysteroids are preferably isolated by means of combined chromatographic methods. The elements of the separation steps applied depend on the nature of the ecdysteroids, and the number of steps has to be increased in accordance with the decreasing concentration of the individual ecdysteroid to be isolated.

Our newly established method, the solvent peak-cut, can be used with ease to improve the separation of the overlapping peaks of isovitexirone **17** and 14-epi-20-hydroxyecdysone **18** and of isovitexirone **17** and ajugasterone C **8**.

Triple conjugation of the 2 double bonds and the oxo group makes possible the specific detection of certain ecdysteroids at 300 nm.

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Legends to the figures

Figure 1. The scheme of ecdysteroid isolation from the prepurified extract

Figure 2. NP-HPLC of a fraction containing herkesterone **15** and 25-hydroxydacryhainansterone **16**. Detection was carried out at 242 nm (**A**) and 300 nm (**B**).
Peak 1: herkesterone, peak 2: 20-hydroxyecdysone, peak 3: 25-hydroxy-dacrychainansterone. The stationary phase was Zorbax SIL® (DuPont, Wilmington, DE, USA) (5 μm), and the mobile phase was dichloromethane – isopropanol – water (125:40:3, v/v/v) with a flow rate of 1 mL/min.

Figure 3. Analytical NP-HPLC of a fraction containing ajugasterone C **8** (peak 1 as shoulder), isovitexirone **17** (peak 2), 14-epi-20-hydroxyecdysone **18** (peak 3), 3-epi-20-hydroxyecdysone **19** (peak 4) and 20-hydroxyecdysone **14** (peak 5). The stationary phase was Zorbax SIL® (DuPont, Wilmington, DE, USA) (5 μm), and the mobile phase was dichloromethane – isopropanol – water (125:40:3, v/v/v) with a flow rate of 0.9 mL/min.

Figure 4. Preparative HPLC of 14-epi-20-hydroxyecdysone **18** (peak 3) from isovitexirone **17** and ajugasterone C **8** (peak 1+2) by the peak-cut method (**A**), and the chromatogram of pure 14-epi-20-hydroxyecdysone **18** (**B**). In the preparative HPLC separation, ajugasterone C **8** and isovitexirone **17** eluted together.
The stationary phase was Zorbax SIL® (DuPont, Wilmington, DE, USA) (5 μm), and the mobile phase was dichloromethane – isopropanol – water (125:40:3, v/v/v) with a flow rate of 0.7 mL/min. Peak D is the eluted peak of the dichloromethane, peak iD the time of the injection.

Figure 5. Analytical HPLC separation of ajugasterone C **8** (peak 1) and isovitexirone **17** (peak 2) by the peak-cut method (**A**) and the chromatogram of pure ajugasterone C **18** (**B**).
The stationary phase was Zorbax SIL® (DuPont, Wilmington, DE, USA) (5 μm), and the mobile phase was dichloromethane – isopropanol – water (125:40:3,v/v/v) with a flow rate of 0.7 mL/min. Peak D is the eluted peak of the dichloromethane, peak iD the time of the injection.

Figure 6. The chemical structures of the isolated ecdysteroids.

Table 1. The TLC characteristics of the isolated ecdysteroids.

Ecdysteroid	Color after spraying with sulfuric acid reagent		R_Fx100										
			NP-TLC					RP-TLC				CN-TLC	
	UV (366 nm)	day light	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.
Polipodine B **1**	violet	turquoise	32	20	25	35	38	48	55	49	66	33	50
Ajugasterone D **2**	violet	yellow	-	59	-	58	-	-	-	-	-	-	-
Dacryhainansterone **3**	red	brown	48	56	55	63	71	10	21	-	34	50	15
Pterosterone **4**	violet	turquoise	-	-	-	49	59	-	-	-	-	-	-
20E 20,22-monoacetonide **5**	violet	turquoise	-	38	-	49	-	-	-	-	-	-	-
Makisterone C **6**	violet	brown	50	49	41	40	57	26	33	30	45	30	27
11α-Hydroxy-poststerone **7**	turquoise	red	-	-	21	30	40	-	-	-	-	-	-
Ajugasterone C **8**	dark red	dark red	35	-	34	35	45	29	34	-	47	35	24
22-Deoxy-20,21-dihydroxyE **9**	violet	brown	-	-	-	-	37	-	-	-	-	-	-
22-Deoxy-20E **10**	orange	orange	26	23	30	34	47	30	34	32	58	31	31
5α-20-Hydroxyecdysone **11**	violet	turquoise	28	23	32	38	38	49	54	50	65	21	46
Makisterone A **12**	violet	purple	-	22	31	38	40	57	49	41	-	28	37
Ecdysone (E) **13**	violet	light yellow	33	27	35	40	41	33	38	35	61	30	30
20-Hydroxyecdysone (20E) **14**	violet	turquoise	24	21	27	30	37	47	56	46	66	26	48
Herkesterone **15**	red	red	26	-	-	25	-	57	54	-	68	21	51
25-Hydroxydacryhainansterone **16**	red	red	21	-	27	28	37	52	54	-	68	24	46
Isovitexirone **17**	violet	green	32	-	37	37	43	39	41	-	50	33	23
14-Epi-20-hydroxyecdysone **18**	violet	turquoise	-	-	-	35	42	-	-	-	-	-	-
3-Epi-20-hydroxyecdysone **19**	violet	turquoise	-	-	27	30	38	-	-	-	-	-	-
22-Epi-20-hydroxyecdysone **20**	violet	turquoise	-	-	-	-	30	-	-	-	-	-	-
Turkesterone **21**	dark red	dark red	10	-	12	14	19	99	68	-	77	8	62
20,26-Dihydroxyecdysone **22**	violet	brown	-	-	-	-	20	-	-	-	-	-	-

20-Hydroxyecdysone **14** = 20E
Ecdysone (E) **13** = (E)

Table 2. HPLC retention time of the isolated ecdysteroids

Ecdysteroid	RP-HPLC			NP-HPLC
	2	4	5	1
Polypodine B **1**	12	22.4		4.4
Ajugasterone D **2**	n.d.	5.7	8.0	
Dacryhainansterone **3**	6,0	9.2	8.4	25.5
Pterosterone **4**	14.7			7.6
20-Hydroxyecdysone 20,22-monoacetonide **5**		16.1	9.2	
Makisterone C **6**	7.8	15,8		15.6
11α-Hydroxy-poststerone **7**	10.7		22.3	3.7
Ajugasterone C **8**	10.5	33.3		
22-Deoxy-20,21-dihydroxyecdysone **9**		53.9	23.6	
22-Deoxy-20-hydroxyecdysone **10**	13	28	15.2	10.1
5α-20-Hydroxyecdysone **11**	12.3	33	24	3.7
Makisterone A **12**	12.4	27.0	17.0	5.3
Ecdysone **13**	12	24.4		10.1
20-Hydroxyecdysone **14**	14.7* 15.6	41	21	4.2
Herkesterone **15**	12.5			3.45
25-Hydroxydacryhainansterone **16**	15.7		26.1	3.5
Isovitexirone **17**	10.6*		14.9	7.4

14-Epi 20-hydroxyecdysone **18**	11.3*		22.1	
3-Epi-20-hydroxyecdysone **19**	13.8*		21.9	
22-Epi-20-hydroxyecdysone **20**	23		25.6	
Turkesterone **21**	26.5		36	
20,26-Dihydroxyecdysone **22**	37.5[a]		47.6	3.8

*flow rate 0,9 mL/min

2[a]: dichloromethane—isopropanol –water (125:50:5, v/v/v)

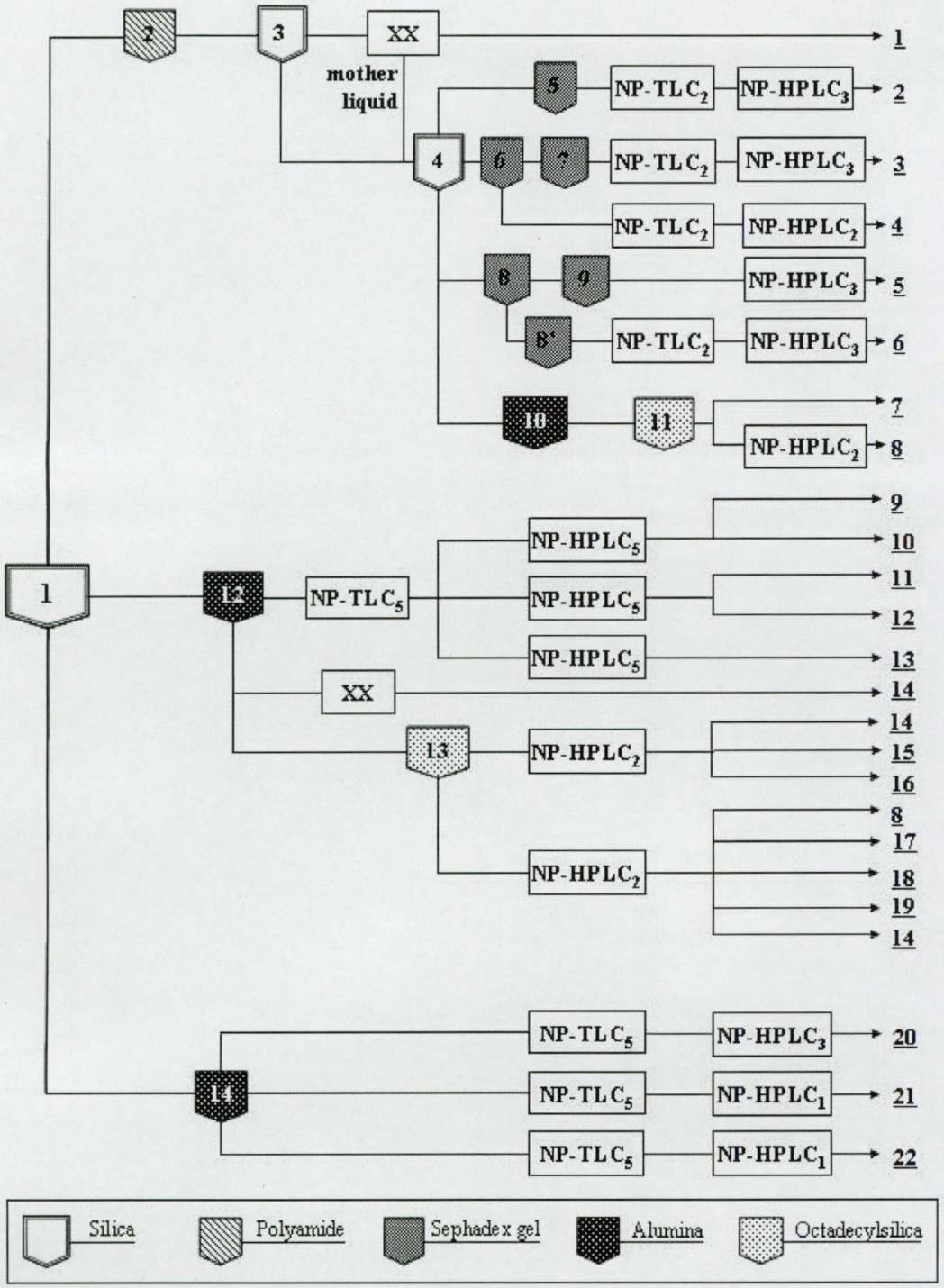
Table 3. The ^{1}H and ^{13}C chemical shifts of the compound 16

No.		16 ^{13}C	^{1}H	m; J (Hz)	No.		^{13}C	^{1}H	m; J (Hz)
1	α	37.5	2.08	dd; 13.5, 4.0	14		84.6	- -	
	β		1.70	dd; 13.5, 11.9	15	a	31.6	1.79	
2	α	68.9	3.72	ddd; 11.9, 3.9, 3.1		b		1.95	
3	α	68.4	3.85	q; 2.8	16	a	22.0	1.80	
4	α	35.9	1.60			b		1.99	
	β		1.76	dt; 14.0, 3.9	17	α	50.7	2.50	t; 8.9
5	β	51.7	2.45	dd; 12.6, 3.7	18	β	18.3	0.90	s
6		*			19	β	31.6	1.11	s
7		119.4	5.75	d; 1.0	20		78.0	- -	
8		156.7	- -		21		21.0	1.211	s
9	(α)	136.3	- -		22		78.6	3.34	overlapped
10		41.1	- -		23	a	27.5	1.31	
11	(α)	134.0	6.29	dt; 6.6, 2.0		b		1.64	
	β				24	a	42.5	1.44	ddd; 13.6, 11.5, 4.3
12	α	39.2	2.74	dd; 17.6, 2.5		b		1.81	
	β		2.42	dd; 18.0, 6.8	25		71.6	- -	
13		48.1	- -		26		29.1	1.19	s
					27		29.8	1.206	s

*: no signal measured

Table 4. Chiroptical data of ecdysteroids

Ecdysteroid	Molar ellipticity					
	λ_1(nm)	[θ]	λ_2(nm)	[θ]	λ_3(nm)	[θ]
Polypodine B **1**	227	22830	256.2	-15930	329.2	9757
Dacryhainansterone **3**	231.8	-3424	253.4 300.8	3648 -15611	345.4	12812
11α-Hydroxy-poststerone **7**	206.2 230.4	-5371 1346	251	-3616	289.4 331.6	2659 1909
Ajugasterone C **8**	207.2 232.4	-15117 12330	257	-10059	330.4	5521
22-Deoxy-20-hydroxyecdysone **10**	223.4	11545	253.6	-17150	330.2	6929
5α-20-Hydroxyecdysone **11**	221	-3757	248.8	-27011	333.2	9457
20-Hydroxyecdysone **14**	224	10047	254	-12883	330	5697
14-Epi-20-hydroxyecdysone **18**			247.4	-9834	328.4	1169
Turkesterone **21**	208 232.6	-6540 5602	257.2	-4525	330.2	2526



Isolated ecdysteroids: Polipodine B **1**, ajugasterone D **2**, dacryhainansterone **3**, pterosterone **4**, 20E 20,22-acetonide **5**, makisterone C **6**, 11α-OH-poststerone **7**, ajugasterone C **8**, 22-deoxy-20,21-dihydroxyecdysone **9**, 22-deoxy-20E **10**, 5α-20E **11**, makisterone A **12**, ecdysone (E) **13**, 20-hydroxyecdysone (20E) **14**, herkesterone **15**, 25-hydroxy-dacryhainansterone **16**, isovitexirone **17**, 14-epi-20E **18**, 3-epi-20E **19**, 22-epi-20E **20**, turkesterone **21**, 20,26-dihydroxyecdysone **22**

Figure 1.

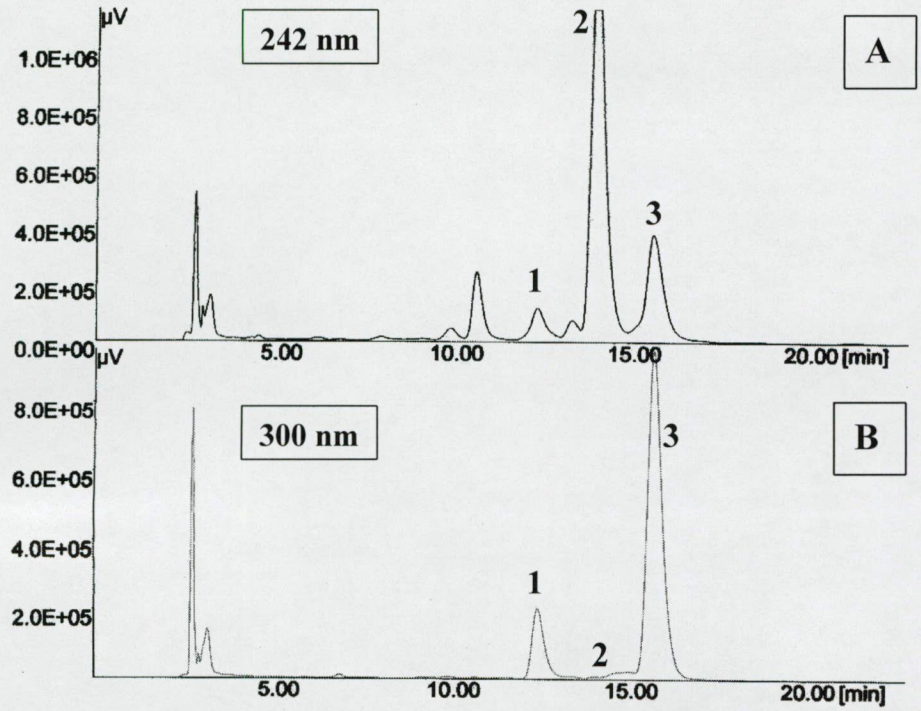


Figure 2.

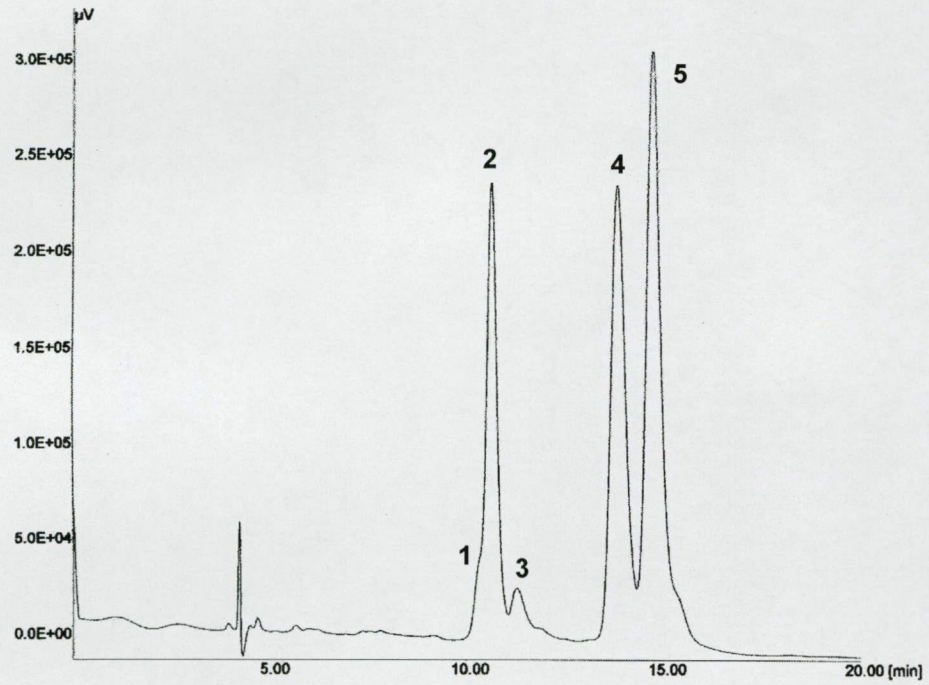


Figure 3.

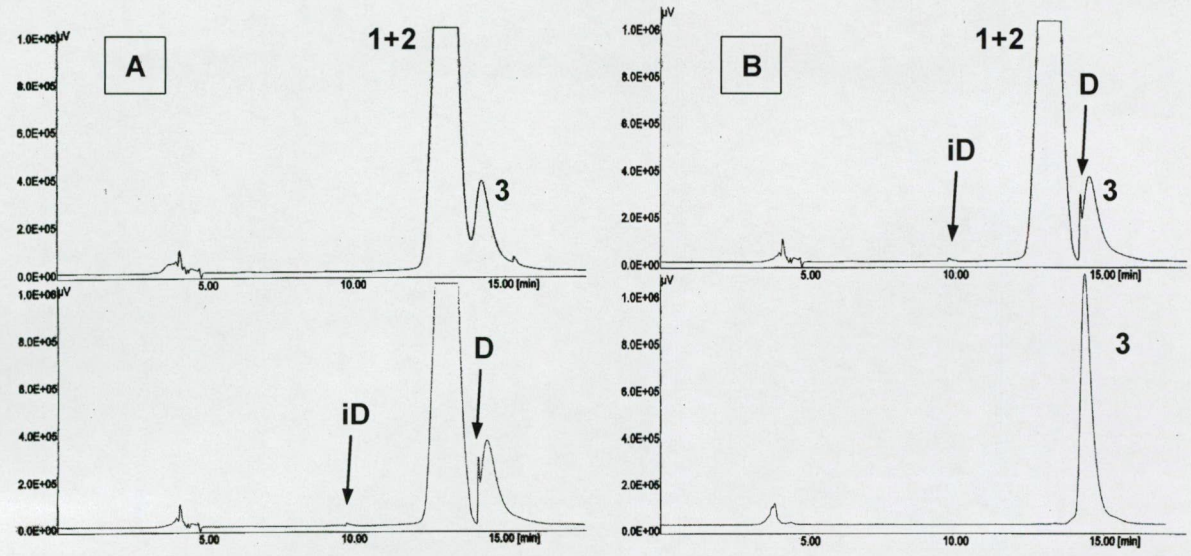
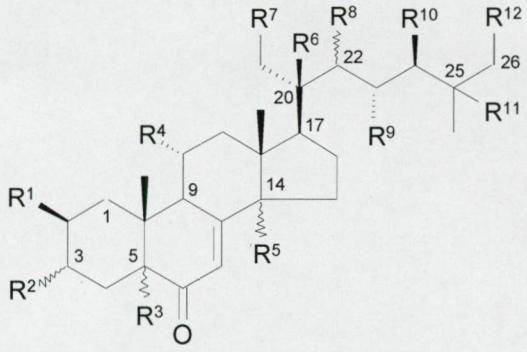


Figure 4.

Figure 5.



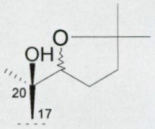
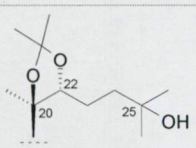
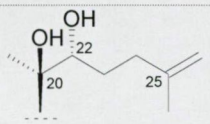
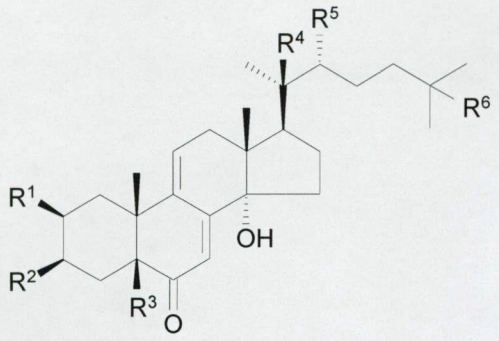
Ecdysteroid	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	R^{10}	R^{11}	R^{12}
Ajugasterone C 8	OH	βOH	βH	OH	αOH	OH	H	αOH	H	H	H	H
Ajugasterone D 2	OH	βOH	βH	H	αOH							
22-Deoxy-20,21-dihydroxy-E 9	OH	βOH	βH	H	αOH	OH	OH	H	H	H	OH	H
22-Deoxy-20E 10	OH	βOH	βH	H	αOH	OH	H	H	H	H	OH	H
20,26-Dihydroxyecdysone 22	OH	βOH	βH	H	αOH	OH	H	αOH	H	H	OH	OH
Ecdysone (E) 13	OH	βOH	βH	H	αOH	H	H	αOH	H	H	OH	H
3-Epi-20E 19	OH	αOH	βH	H	αOH	OH	H	αOH	H	H	OH	H
14-Epi-20E 18	OH	βOH	βH	H	βOH	OH	H	αOH	H	H	OH	H
22-Epi-20E 20	OH	βOH	βH	H	αOH	OH	H	βOH	H	H	OH	H
5α-20E 11	OH	βOH	αH	H	αOH	OH	H	αOH	H	H	OH	H
20-Hydroxyecdysone (20E) 14	OH	βOH	βH	H	αOH	OH	H	αOH	H	H	OH	H
20E 20,22-monoacetonide 5	OH	βOH	βH	H	αOH							
11α-hydroxyposttsterone 7	OH	βOH	βH	OH	αOH							
Isovitexirone 17	OH	βOH	βH	OH	αOH							
Makisterone A 12	OH	βOH	βH	H	αOH	OH	H	αOH	H	CH_3	OH	H
Makisterone C 6	OH	βOH	βH	H	αOH	OH	H	αOH	H	C_2H_5	OH	H
Polipodine B 1	OH	βOH	βOH	H	αOH	OH	H	αOH	H	H	OH	H
Pterosterone 4	OH	βOH	βH	H	αOH	OH	H	αOH	H	αOH	H	H
Turkesterone 21	OH	βOH	βOH	OH	αOH	OH	H	αOH	H	H	OH	H

Figure 6/A Structures of the isolated ecdysteroids in alphabetical order (Part 1.)



Ecdysteroid	R^1	R^2	R^3	R^4	R^5	R^6
Dacryhainansterone 3	OH	OH	H	OH	OH	H
Herkesterone 15	OH	OH	OH	OH	OH	OH
25-hydroxy-dacryhainansterone 16	OH	OH	H	OH	OH	OH

Figure 6/B Structures of isolated ecdysteroids (Part 2.).