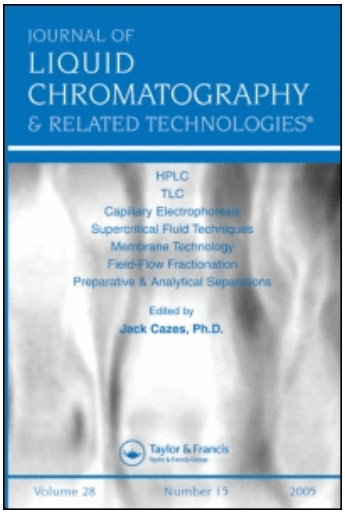

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Role of Preparative Rotation Planar Chromatography in the Isolation of Ecdysteroids

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To cite this Article Kalász, Huba , Liktor-Busa, Erika , Janicsák, Gábor and Báthori, Mária(2006) 'Role of Preparative Rotation Planar Chromatography in the Isolation of Ecdysteroids', Journal of Liquid Chromatography & Related Technologies, 29: 14, 2095 — 2109

To link to this Article: DOI: 10.1080/10826070600759967

URL: http://dx.doi.org/10.1080/10826070600759967

Journal of Liquid Chromatography & Related Technologies®, 29: 2095–2109, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600759967

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Abstract: A reliable isolation procedure is elaborated for the purification of ecdysteroids from *Serratula wolffii*. The procedure is also applicable to other plant sources. The general ecdysteroid isolation procedure was improved by the introduction of preparative rotation planar chromatography (RPC) to the purification process. Effective and simple cleanup and vacuum reversed-phase column chromatographic separation was completed with RPC, or repeated RPC, to obtain pure ecdysteroids (ajugasterone C, dacryhainansterone, 22-deoxy-integristerone A, 20-hydroxyecdysone, and 2 new ecdysteroids) from the crude extract. This paper discusses the advantages of this method as the final step of ecdysteroid isolation.

Keywords: Ecdysteroids, Plant material, *Serratula wolffii*, Rotation planar chromatography

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INTRODUCTION

Ecdysteroids occur in plants in multicomponent mixtures of structurally related compounds.[1,2] Their isolation generally involves a complex combination of a series of preparative-scale chromatographic procedures, such as thin-layer chromatography, normal- and reversed-phase column chromatography, flash chromatography, droplet counter-current chromatography (DCCC), gel chromatography, and high performance liquid chromatography (HPLC).[3–5]

There has recently been a great interest in ecdysteroids because of their uses in both traditional and modern medicine and agriculture.[6,7] The ecdysteroids exert molting hormone activity to insects and various forms of significant pharmacological activities to mammals, including humans. Their anabolic action without thymolytic and androgenic side-effects is the most thoroughly investigated and scientifically proved[8] aspect of their pharmacological behavior.

Ecdysteroids have also attracted interest as inducers of gene-regulation systems based on the ecdysone receptors of insects. Ecdysteroid 7,9(11)-dienones seem to be suitable ecdysteroid receptor agonists, as they exert high hormone activity on insects binding with high affinity to the ecdysteroid receptor.[9]

The pharmacological and biological importance of ecdysteroids has initiated attempts to improve their isolation procedures. The *Serratula* plant species are rich sources of ecdysteroids. *S. wolffii* is characterized by a high accumulation and wide structural diversity of ecdysteroids. These species biosynthesize a series of biologically active ecdysteroids, among them 11-hydroxylated ecdysteroids, and ecdysteroids with 7,9(11)-dienone structures. 11-Hydroxylation is important for manifestation of the anabolic action of ecdysteroids.[10]

The aim of the present work was to improve and simplify the procedure of ecdysteroid isolation and to study the role of rotation planar chromatography in this process. This paper follows the nomenclature of planar chromatography outlined by Nyiredy et al.,[11] so RPC is the abbreviation of rotation planar chromatography, not reversed-phase chromatography. The effective cleanup and optimized combination of vacuum reversed-phase column chromatography on octadecyl silica and repeated preparative rotation planar chromatography on silica resulted in pure, biologically active ecdysteroids, such as ajugasterone C, dacrychainansterone, 22-deoxy-integristerone A, 20-hydroxyecdysone, and two earlier unknown ecdysteroids. The ecdysteroids were identified using thin-layer chromatography (TLC) and mass spectrometry (MS).

EXPERIMENTAL

Plant Material

Roots of *S. wolffii* Andrae were collected in August, 2003 from Herencsény, Hungary. A voucher specimen (collection number S94) was deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

TLC

TLC was carried out on 20 × 20 cm glass plates coated with silica gel F_{254} (E. Merck, Darmstadt, Germany). The plates were developed by the ascending technique in an unsaturated glass chamber (Desaga, Heidelberg, Germany) at room temperature. The following mobile phases were used:

- dichloromethane-methanol-benzene (50:10:6 v/v/v);
- ethyl acetate-96% ethanol-water (80:10:5 v/v/v).

The plates were developed to a distance of 148 and 150 mm (used for the separation of ajugasterone C). After development of the plates, the ecdysteroids were detected either directly by fluorescence extinction at 254 nm or by the use of vanillin-sulfuric acid spray reagent. After spraying, the spots were observed either in daylight or at 366 nm.

Densitograms were recorded using a Shimadzu CS-9301PC densitometer (Osaka, Japan) in the reflectance-absorbance mode at 254 nm.

RPC

A Harrison Model 8924 Chromatotron instrument (Harrison Research, Palo Alto, CA) was used. The stationary phase was silica gel 60 GF_{254} (E. Merck), manually coated on the rotor as a 1 mm layer. Previous TLC experiments served as a tentative guide for mobile phase selection. Stepwise development was used with nine mobile phases (100 mL each):

- System 1/A: chloroform-methanol-benzene (50:3:2 v/v/v);
- System 1/B: chloroform-methanol-benzene (50:5:3 v/v/v);
- System 1/C: chloroform-methanol-benzene (50:10:6, v/v/v);
- System 2/A: ethyl acetate-ethanol-water (80:2:1, v/v/v);
- System 2/B: ethyl acetate-ethanol-water (80:5:2, v/v/v);
- System 2/C: ethyl acetate-ethanol-water (80:10:5, v/v/v);
- System 3/A: ethyl acetate-ethanol-water (80:5:2, v/v/v);
- System 3/B ethyl acetate-ethanol-water (80:7:3, v/v/v);
- System 3/C: ethyl acetate-ethanol-water (80:10:5, v/v/v).

Before applying the sample, the dry stationary phase was completely wetted with the firstly applied mobile phase (either solvent system 1/A or 2/A, 3/A, 50 mL in each case), and a further 5 min was allowed for equilibration. The solution of the sample (see below) dissolved in the first elution solvent (3 mL) was introduced through the inlet. The mobile phase flow rates were 4–5 mL min^{-1} (see below); in each case, thirty 10 mL fractions were collected. Therefore, the total elution times were 60 min and 70 min. The sorbent layers were regenerated with 50 mL of methanol. The separation

was monitored by observing under ultraviolet (UV) illumination at 254 nm and by TLC, and the separation of the fractions was verified by normal-phase TLC.

Vacuum Reversed Phase Chromatography (RP-CC)

Vacuum RP-CC was carried out on end-capped octadecyl silica (0.06–0.2 mm particle size) (Chemie Urticon-C-gel, C-560, Ueticon, Switzerland) packed into a 400 × 32 mm glass column. Stepwise gradient elution was done using methanol-water (30:70, 35:65, 40:60, 45:55, 50:50, 55:45, and 60:40 v/v); 1,000 mL each.

Solid Phase Extraction (SPE)

The stationary phase MN-polyamide SC 6 for column chromatography, 0.06–0.16 mm (Woelm, Eshwege, Germany) was used for the cleanup. The ecdysteroids were eluted with deionized water.

Ecdysteroid Isolation

The fresh roots of *S. wolffii* (4763 g) were washed, milled, and percolated with 20 L of methanol at room temperature. The extract was evaporated, the dry residue (208.9 g) was dissolved in 800 mL of methanol, and 400 mL of acetone was then added to the solution. The formed precipitate was separated by decantation and was washed three times with 100 mL of methanol-acetone (2:1 v/v) mixture. The methanol-acetone solutions were combined and evaporated to dryness, and the residue was dissolved in 700 mL of methanol. The precipitation was repeated twice, using 700 mL and 1,200 mL of acetone. The final residue of the methanol-acetone solution, obtained after the fractionated precipitation (137.5 g), was dissolved in 300 mL of methanol, adsorbed onto polyamide stationary phase, and packed into the top of a column containing 344 g polyamide (210 × 145 mm). The ecdysteroids were eluted from the polyamide with 2 L of water. The aqueous solution was evaporated, and the dry material (24.4 g) dissolved in 50 mL of methanol.

Vacuum RP-CC was carried out in two parallel procedures. Half of the methanolic solution was applied to the top 150 g of end-capped octadecyl silica packed in the column. Elution from the column was carried out with stepwise gradients of 30%, 35%, 40%, 45%, 50%, 55%, and 60% aqueous methanol (1,000 mL each) at a flow rate of $5\,mL \cdot min^{-1}$, and 200 mL fractions were collected. Fractions 18–23 were combined and evaporated to dryness. The dry residue (0.39 g) was dissolved in 3 mL of RPC developing

solvent 1/A and applied to the middle of the rotation plate after equilibration. The plate was developed stepwise with three mobile phases (solvents 1/A, 1/B, and 1/C). The effluents were collected manually in test tubes.

The contents of each fraction were checked using conventional TLC. The fractions containing the same composition were combined and evaporated to dryness. The number of combined fractions was at least nine, and these were again investigated by TLC. The combined fractions A, C, E (derived from fractions 5–6, 10–14, and 24–25, respectively) were further purified by crystallization.

The combined fractions B (derived from fractions 7–8, dry residue 17 mg) and D (derived from fractions 18–23, dry residue 48 mg) were fractionated again by RPC using solvent systems 2/A, 2/B, and 2/C. The RPC separation was carried out similarly as above, but the mobile phase flow rate was now 4 mL min^{-1}. The contents of each fraction were controlled by conventional TLC. The fractions containing the same compounds were combined and evaporated to dryness. In the first case (combined fractions B), the repeated RPC resulted in pure dacryhainansteron (1 mg) in fraction 2. In the second case (combined fractions D), two pure ecdysteroids were obtained, a new ecdysteroid (ecdysteroid 1, 15 mg) and ajugasterone C (14 mg). According to the TLC measurements, these ecdysteroids were present in fractions 3–6 and 11–12, respectively.

The methanolic washing solution obtained during regeneration of the plate (50 mL) was further purified by RPC in the same way, using solvent systems 3/A, 3/B, and 3/C, with a mobile phase flow rate of 4 mL min^{-1}. Fractions 14–18 contained pure 22-deoxy-integristerone A (3 mg).

RESULTS

Our isolation procedure involved two main steps:

1. Extraction and cleanup of the crude extract using simple non-chromatographic methods.
2. Separation of the ecdysteroids by using preparative-scale chromatographic techniques, such as vacuum RP-CC and preparative RPC on silica.

Figure 1 shows the isolation procedure of ecdysteroids.

The ecdysteroids were subjected to exhaustive extraction with methanol in a percolator at a solvent:plant ratio of 7:1. The extraction resulted in an eight-fold purification. The first step of the cleanup involved fractionated precipitation with acetone, as described earlier.[3] The consecutive precipitation steps removed the overwhelming majority of the polar contaminants. For this, the crude methanolic extract was mixed with acetone in extract:acetone volumetric ratios of 2:1, 1:1, and 1:2. The resulting acetone-methanolic

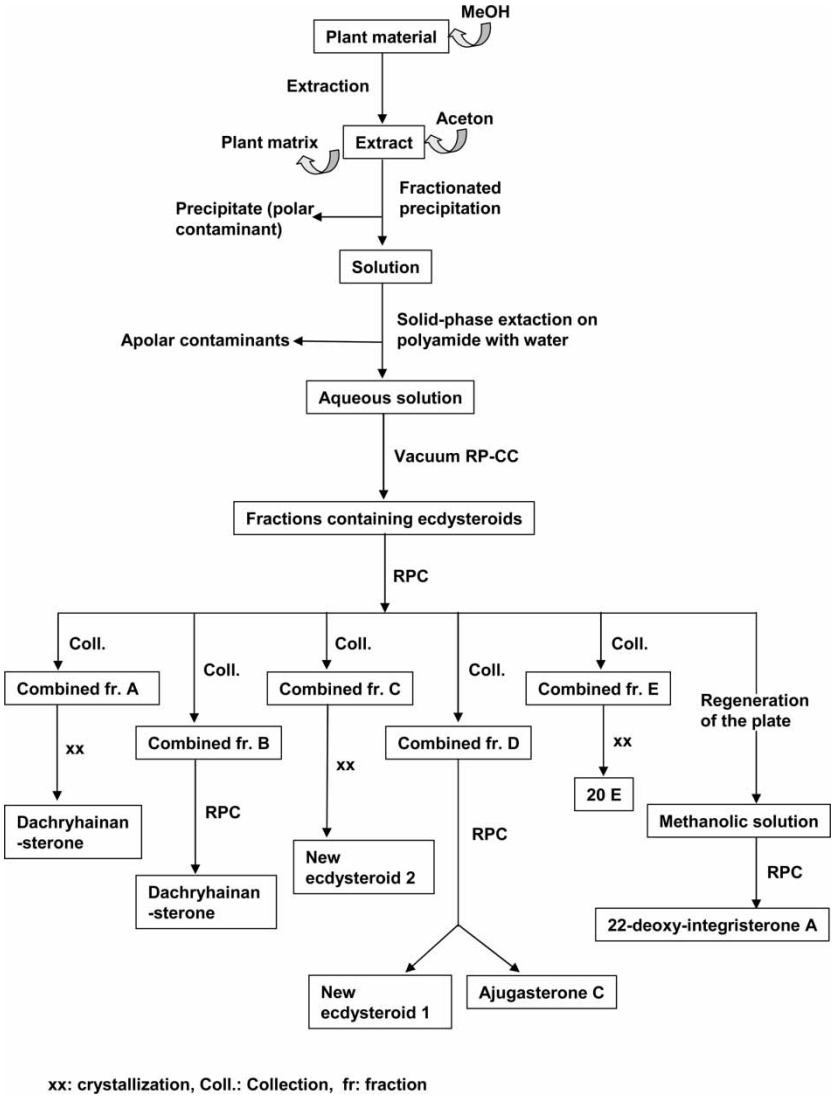

Figure 1. Isolation procedure of ecdysteroids.

solution contained the ecdysteroids, while the precipitate consisted of the impurities. The next cleanup step was by SPE on polyamide, carried out on a home-made set-up, in which 344 g of polyamide was loaded with 137.1 g of sample. The sample was adsorbed onto the polyamide, and the ecdysteroids were eluted from the sorbent with water. The impurities, mainly phenoloids, remained adsorbed on the polyamide. The overall cleanup procedure resulted in a 5-fold purification.

After the cleanup, vacuum RP-CC on octadecyl silica resulted in a crude separation of the prepurified extract. The fractions obtained by RP-CC contained complex mixtures of structurally related ecdysteroids. The ecdysteroids of interest were eluted with 45% and 50% aqueous methanol (Figure 2). These fractions were combined and further purified by preparative RPC on silica.

In the first RPC separation, 42 g of stationary phase was loaded with 390 mg of dry sample, giving an adsorbent-sample ratio of 100:1. Fractionation was carried out with stepwise gradient elution (solvent systems 1/A-1/C) in three steps. The starting mobile phase was chosen by decreasing the solvent strength employed in conventional analytical TLC. The fractionation was controlled by conventional TLC. The fractions containing the same ecdysteroids were combined. TLC analysis has shown that RPC in a single run resulted in three almost pure ecdysteroids: combined fractions A contained dacryhainansterone, combined fractions E 20-hydroxyecdysone, and combined fractions C an earlier unknown new ecdysteroid in almost pure form (Figure 3). To obtain spectroscopically pure ecdysteroids from these fractions, simple crystallization was done.

Combined fractions B contained a mixture of five ecdysteroids (Figure 4A), among which dacrychainanstreone was the main component. These ecdysteroids were further purified by the repeated use of RPC employed with solvent systems with different selectivities (solvent systems 2/A–C). The RPC was carried out as described above. Dacrychainanstreone was eluted at the beginning of the separation with solvent system 2/A (Figure 4B).

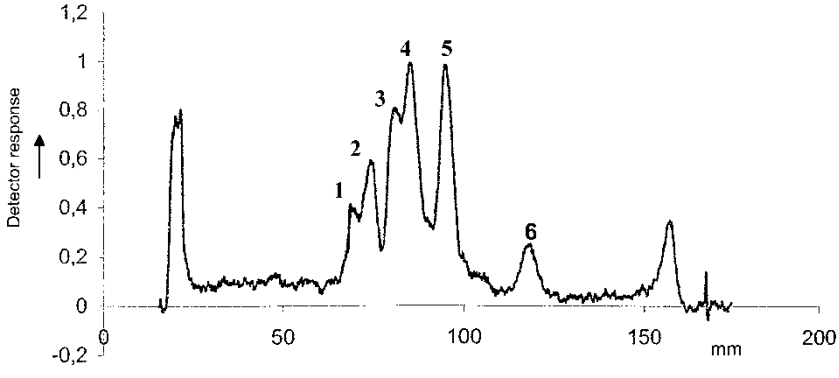

Figure 2. Densitogram of an RP-CC fraction containing the ecdysteroids of interest. Peaks: *1* = 22-deoxy-integristerone A; *2* = 20-hydroxyecdysone; *3* = new ecdysteroid 1; *4* = new ecdysteroid 2; *5* = ajugasterone C; and *6* = dacrychainansterone. Stationary phase: silica gel. Mobile phase: ethyl acetate–96% ethanol - water (80:10:5 v/v/v).

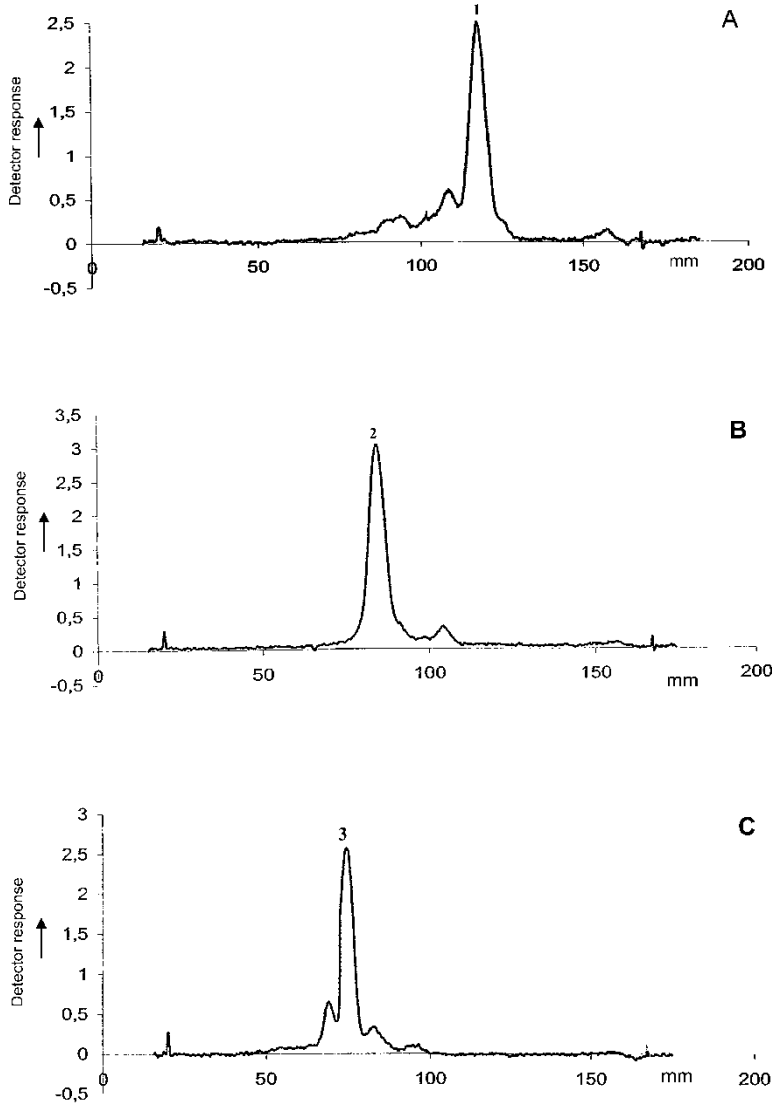

Figure 3. Densitogram of the combined fractions A, C, and E obtained in the first RPC separation. Peaks: *1* = dacrychainansterone (Figure 3A); *2* = new ecdysteroid 2 (Fig. 3B); *3* = 20-hydroxyecdysone (Figure 3C). Stationary phase: silica gel. Mobile phase: ethyl acetate–96% ethanol – water (80:10:5 v/v/v).

Ajugasterone C was eluted in the first RPC separation with solvent systems 1/B and 1/C, together with another main compound (combined fractions D) (Figure 5A). Repeated RPC resulted in a new ecdysteroid (solvent system 2/A, Figure 5B) and pure ajugasterone C (solvent system 2/B, Figure 5C).

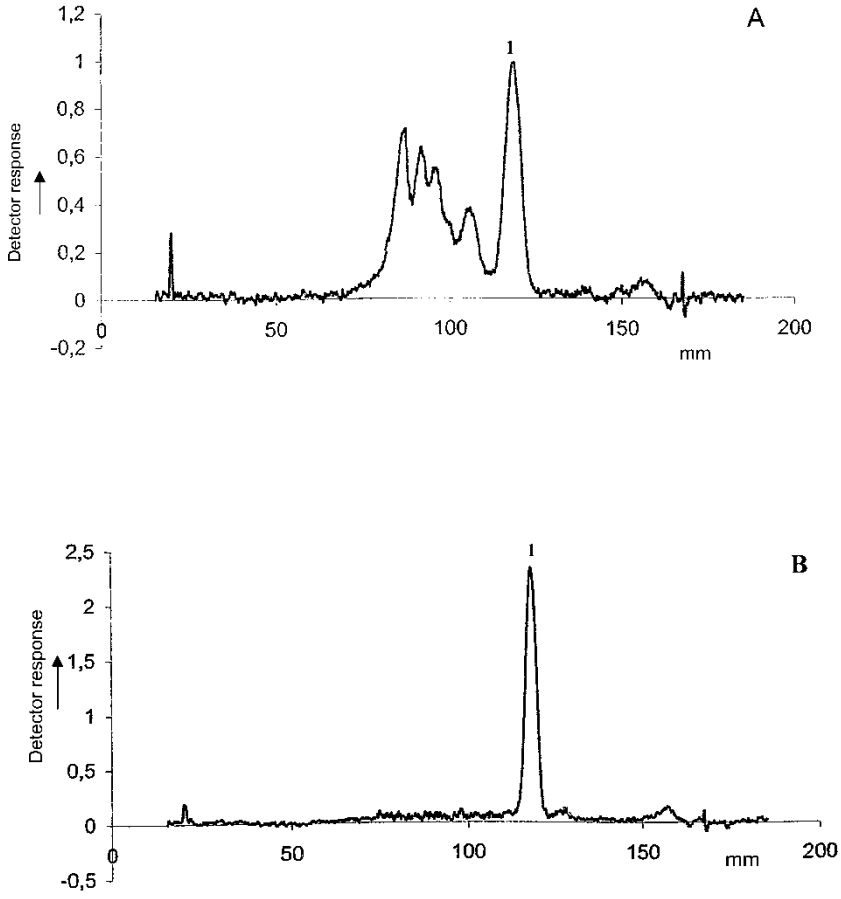

Figure 4. Densitogram of the dacrychainansterone-containing fraction (combined fractions B) obtained in the first RPC separation (Figure 4A) and densitogram of pure dacrychainansterone obtained from this fraction by repeated RPC (Figure 4B). Peak *1* = dacrychainansterone. Stationary phase: silica gel. Mobile phase: ethyl acetate – 96% ethanol–water (80:10:5 v/v/v).

After the fractionation in the first RPC process, a polar ecdysteroid (22-deoxy-integristerone A) remained adsorbed on the stationary phase, and its elution required the use of a polar solvent such as methanol. The use of 50 mL of methanol mm^{-1} of layer thickness was sufficient for the desorption of this ecdysteroid, and 22-deoxy-integristerone A eluted together with some impurities (Figure 6A). The impurities were removed by a repeated RPC process with solvent systems 3/A-3/C to obtain pure 22-deoxy-integristerone A (Figure 6B). The compound was eluted in the middle of this fractionation when solvent system 3/B was in use.

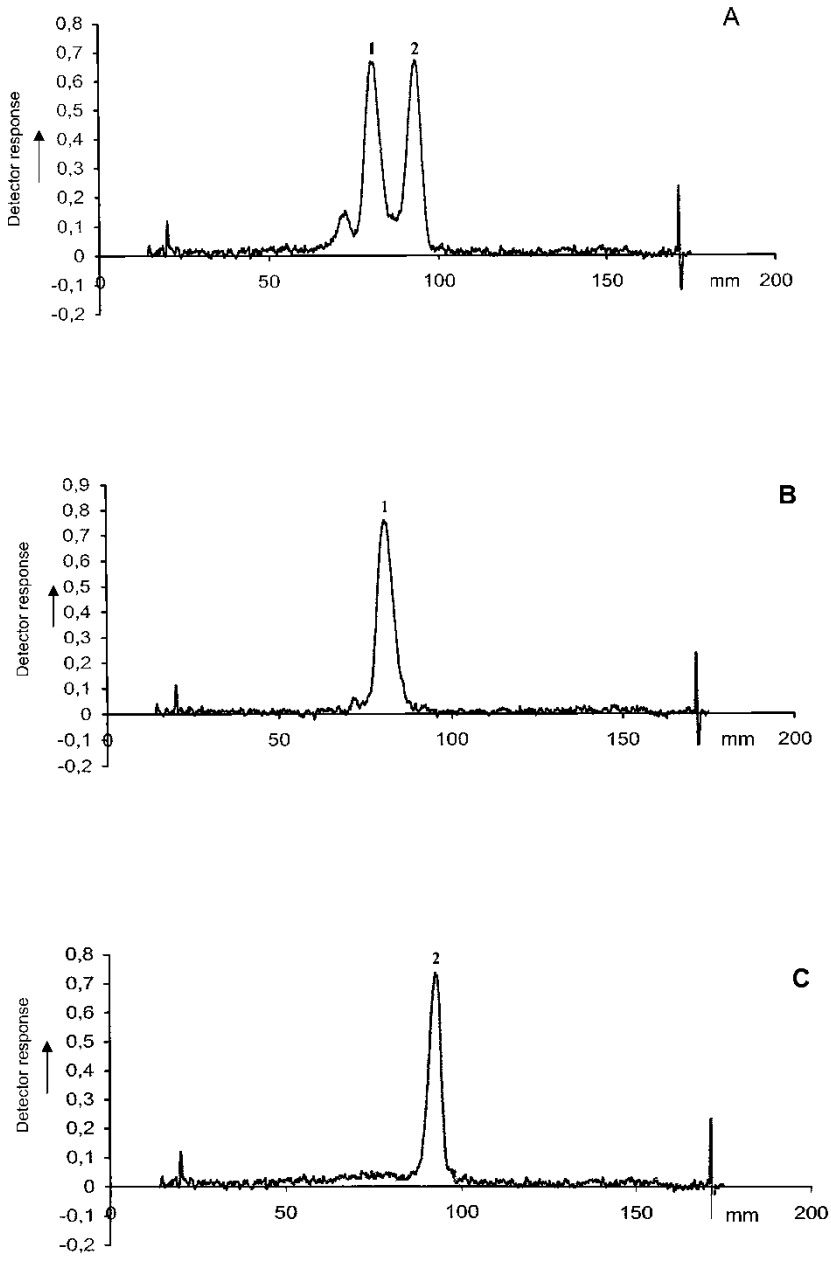

Figure 5. Densitogram of the ajugasterone C-containing fraction (fraction D) obtained in the first RPC separation (Figure 5A) and densitogram of a new ecdysteroid and pure ajugasterone C obtained from this fraction by repeated RPC (Figure 5B and 5C, respectively). Peaks: *1* = new ecdysteroid 1; *2* = ajugasterone C. Stationary phase: silica gel. Mobile phase: ethyl acetate–96% ethanol–water (80:10:5 v/v/v).

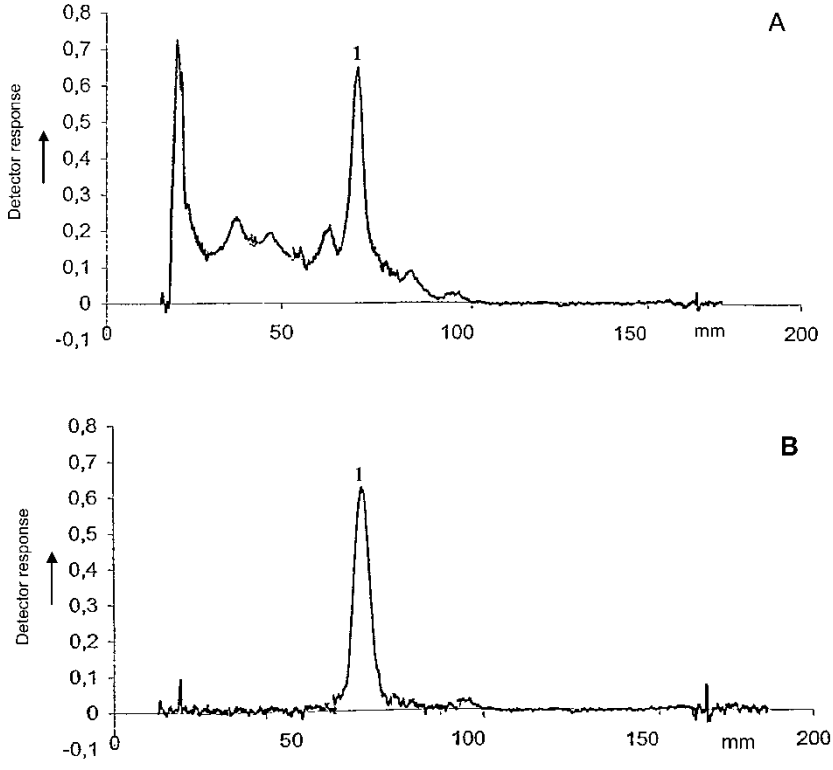

Figure 6. Densitogram of the solution obtained by the regeneration of the rotation plate after the first RPC separation (Figure 6A) and densitogram of pure 22-deoxy-integristerone A obtained from this solution by repeated RPC (Figure 6B). Peak *1* = 22-deoxy-integristerone A. Stationary phase: silica gel. Mobile phase: ethyl acetate – 96% ethanol – water (80:10:5 v/v/v).

The chemical structures of the newly isolated ecdysteroids have been determined by spectroscopic methods (to be published later). The known ecdysteroids were identified by TLC and MS; the compounds were identical to the reference compounds, they migrated together, and they gave the same molecular mass peak and fragments. Figure 7 shows the structures of the isolated known ecdysteroids.

DISCUSSION

As described, a sophisticated strategy was developed for the isolation of ecdysteroids from *S. wolffii*. Their isolation from the partially purified plant extract was based on a combination of vacuum RP-CC and RPC. Our aim in using

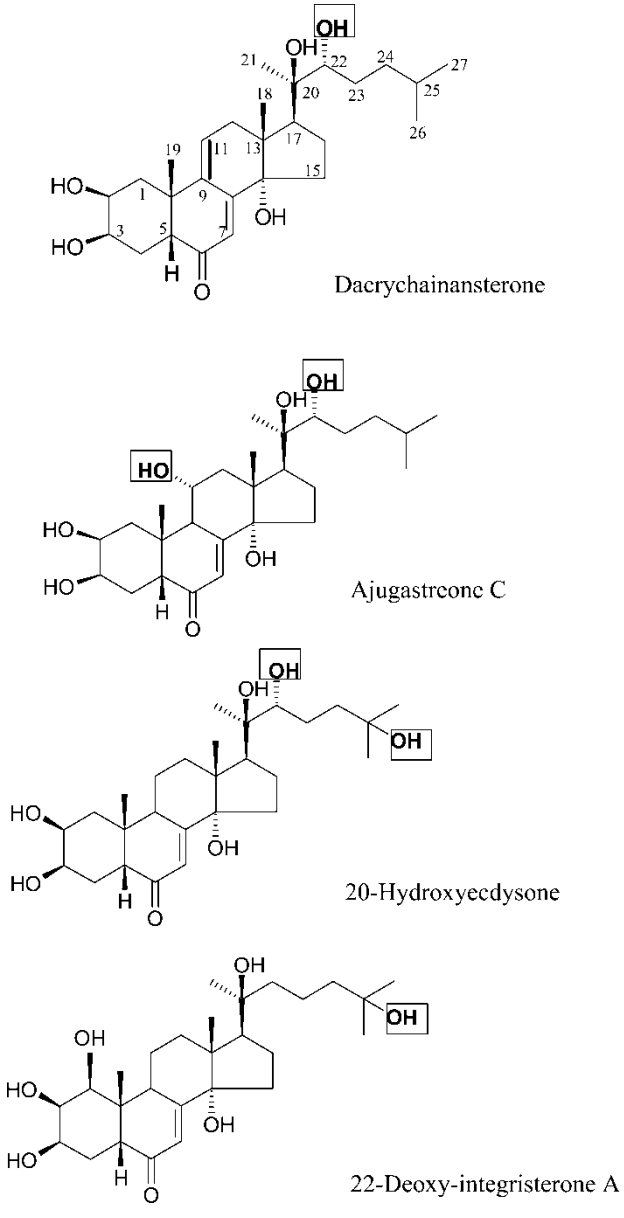

Figure 7. The structures of the isolated known ecdysteroids.

RPC was to develop a new method for the rapid separation of ecdysteroids. Earlier isolation methods consisted of several absorption chromatographic steps, with both column and planar techniques and DCCC, while preparative TLC and/or HPLC was used for final purification. In this new isolation

process, the use of only two chromatographic methods proved sufficient to obtain pure ecdysteroids. Based on the different physical-chemical characteristics of the ecdysteroids, RPC and RP-CC applied in the consecutive steps provided different selectivities and improved resolution. Here, in the final purification step, TLC and HPLC have been replaced by RPC. The earlier separation procedure[2–5] was improved and simplified by the use of RPC on silica, together with RP-CC.

Preparative, centrifugally accelerated, RPC achieved with the Chromatotron instrument ensured good separation of the ecdysteroids from *S. wolffii*. This procedure is generally applicable to other plant sources also.

Several conditions, such as the layer thickness, different mixtures of solvent systems, and the solvent flow rate, which depends on the rotation speed, were adjusted to achieve the best separation.[11–13] It was established that pre-wetting of the silica plate was required for successful separation. A lower flow rate (<4 mLmin^{-1}) did not lead to an improved resolution. RPC gave almost complete separation of three ecdysteroids in a single run from the fractions obtained by prepurification and RP-CC on the crude *S. wolffii* extract. The partial separation in one pass was completed by a repeated pass with another optimized mobile phase, with different selectivity.

The elution order of the ecdysteroids with six hydroxyl groups was unusual when RPC with solvent systems 1/A-1/C were used: ajugasterone C, containing a hydroxyl group at position 11, was eluted earlier than 20-hydroxyecdysone and 22-deoxy-integristerone A. The latter two ecdysteroids are hydroxylated at position 25 (Figure 7). This shows that the chromatographic behavior of the ecdysteroids is strongly affected by the positions of the hydroxy groups.[14,15] Hydroxylation at position 25 has a pronounced effect on the chromatographic characteristics because this hydroxyl group is located in a hydrophobic part of the molecule. It is interesting that 22-deoxy-integristerone A was eluted later than 20-hydroxyecdysone, which has an additional hydroxyl group on the apolar side-chain at position 22.

The use of RPC as a final purification step in ecdysteroid isolation offers some advantages:

- RPC is easier to carry out than the conventional preparative TLC separation.[16]
- The ecdysteroids are in contact with the adsorbent layer for a shorter time than in TLC. Therefore, the problems associated with adsorbent-assisted decomposition are reduced.
- RPC is an on-line preparative chromatographic method, newly introduced for ecdysteroid isolation. It is a simple forced-flow technique driven by centrifugal force. Therefore, this procedure is faster than preparative TLC and provides better separation. RPC is an effective, inexpensive tool for the separation of ecdysteroids in a complex mixture with low solvent usage and less time consumption.
- After one cleaning with methanol, the silica gel layer gave sharper bands.

- RPC permits a larger loading capacity and favorable operating simplicity as compared to HPLC.
- RPC is a very convenient procedure in the final purification steps of ecdysteroid isolation, when 250 mg–1.5 g sample must be separated.

This newly developed purification procedure has led to the isolation of two previously unknown ecdysteroids and several known, biologically important ecdysteroids, such as ajugasterone C, dacrychainansterone, 22-deoxy-integristerone A, and 20-hydroxyecdysone.[6,7] 20-Hydroxyecdysone is the main ecdysteroid of plants and possesses several scientifically proven pharmacological effects. Ajugasterone C is an 11-hydroxylated ecdysteroid, while dacrychainansterone has a 7,9(11)-dienone structure; they attract attention as anabolic and receptor agonists.

ACKNOWLEDGMENT

The advice of Dr. L.S. Ettre is highly appreciated.

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Received January 14, 2006
Accepted February 15, 2006
Manuscript 6864A