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# Preparative Scale Purification of Shidasterone, 2-Deoxy-polypodine B and 9α,20-Dihydroxyecdysone from *Silene italica* ssp. *nemoralis*

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## Abstract

**A suitable combination of preparative scale separation methods results in effective clean-up of the ecdysteroids of *Silene italica* ssp. *nemoralis* (Waldst. and Kit.) Nyman. The isolation of minor ecdysteroids from the partially purified extract is based on the use of both droplet counter-current chromatography and low-pressure reversed-phase liquid chromatography. The purification is completed by preparative thin-layer chromatography and preparative high-performance liquid chromatography to obtain the minor ecdysteroids, such as 2-deoxy-20-hydroxyecdysone, shidasterone, 2-deoxy-polypodine B, makisterone C, and 9α,20-dihydroxyecdysone.**

## Introduction

Over 250 chemically unique ecdysteroids have been identified from various plant species. Their beneficial pharmacological effects and potential application in genetics promotes interest in these compounds and their isolation (1). The number of identified phytoecdysteroids is still slowly increasing because of the progress in the isolation and structure elucidation methods. Until now, the studies have been dealing with the plant species in specific families containing large amounts of ecdysteroids with wide structural diversity. The main ecdysteroids and a large array of the minor ecdysteroids of these plants became known. Search for hitherto unknown ecdysteroids should be focused on the trace constituents of plant species.

Chromatography has been the key method to find new minor ecdysteroids. As the search has been dealing with ecdysteroids present in lower and lower concentration level in the plants, the isolation system has to be more and more effective. A few obstacles can hinder the isolation of minor ecdysteroids from a complex mixture of other compounds present in the plant extract: minor ecdysteroids produced in plants at a trace level, such as under 0.001%. However, structural elucidation requires approximately 1 mg of the pure compound (2). The direct outcome of this requirement is that at least 1 kg of the dried plant has to be subjected to a multistep procedure of either production-scale or preparative scale separation. The complexity of the isolation procedure is determined by the nature and amounts of accompanying substances (nonecdysteroid contaminants). The wide diversity of structurally related ecdysteroids in the plant extracts (3) can also play a fundamental role in the multiplicity of the isolation protocol. The last point to be considered is the high excess of the main ecdysteroid (20-hydroxyecdysone) to the minor ecdysteroids of interest. Its selective separation is an important requirement for the successful isolation of minor ecdysteroids, otherwise 20-hydroxyecdysone would contaminate the entire chromatographic system (4).

This paper reports on a general process for the isolation of minor ecdysteroids. Isolation of five minor ecdysteroids from *Silene italica* ssp.*nemoralis* is reported on. The method can also be adopted to the purification of minor ecdysteroids of other plant material. The isolated new ecdysteroids represent lead compounds for the synthetic and semisynthetic preparation and can been used in structure-activity relationship study.

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## Experimental

### Materials

*Plant material*

The aerial parts of Silene italica ssp. nemoralis (Waldst. and Kit.) Nyman were collected in the Botanical Garden of Vácrátót (Vácrátót, Hungary) on May 26, 1997. A voucher specimen was deposited at the Department of Pharmacognosy, University of Szeged (Szeged, Hungary).

*Chemicals*

Solvents and chemicals were purchased from commercial sources in the best available quality. The standard ecdysteroids

were isolated in our earlier experiments (5).

Solid-phase extraction (SPE) used Alumina Brockmann II neutral (Reanal, Budapest, Hungary) (300- × 93-mm, i.d.). Reversed-phase (RP) low-pressure (LP) column chromatography (CC) was carried out on Kovasil $C_{18}$ (0.06–0.2-µm particle size, Chemie Uetikon, Uetikon, Switzerland, 187 g) packed in a 3.5- × 42-cm column, and the entire procedure was carried out at LP drop (< 1 atm).

### Thin-layer chromatography

Normal-phase (NP) thin-layer chromatography (TLC) was carried out on 20- × 20-cm Silica gel 60 $F_{254}$ (Cat. No. 1.05554, Merck, Darmstadt, Germany) TLC plates. The following solvent mixtures were used as the mobile phase: toluene–acetone–ethanol (96%)–ammonia (25%) (100:140:32:9, v/v/v/v) (I), dichloromethane–methanol–benzene (25:5:3, v/v/v) (II), ethyl acetate–ethanol (96%)–water (16:2:1, v/v/v) (III), dichloromethane–ethanol (96%) (8:2, v/v) (IV), and ethyl acetate–methanol–ammonia (25%) (85:10:5, v/v/v) (V). RP-TLC employed 20- × 20-cm RP-18 $F_{254S}$ (Cat. No. 1.15389, Merck) TLC plates with the following mobil phases: acetonitrile–trifluoroacetic acid (0.1%) (25:75 and 35:65, v/v) (VI and VII), ethyl acetate–formic acid–water (85:10:5, v/v/v) (VIII), methanol–water (6:4, v/v) (IX), and tetrahydrofuran–water (45:55, v/v) (X). The spots were visualized both by fluorescent quenching at 254 nm, and in the case of NP-TLC, after spraying with vanillin-sulfuric acid and observing in daylight or at 366 nm. For the preparative forms of NP-TLC, 20 × 20 Silica gel 60 $F_{254}$ (Cat. No. 1.05715, Merck) TLC plates were used. The bands were located by UV detection as described previously. The bands of compounds were scraped after development, and the ecdysteroids were extracted from the stationary phase using methanol.

### High-performance liquid chromatography

The system used for high-performance liquid chromatography (HPLC) consisted of a Shimadzu LC-10AS pump and a SPD-10A Shimadzu UV–vis detector. For analytical NP-HPLC, a Zorbax-SIL column (5 µm, 250- × 4.6-mm i.d.) (DuPont, Paris, France) was used and eluted with either dichloromethane–isopropanol–water (125:40:3 and 125:30:2, v/v/v) (XI and XII) or cyclohexane–isopropanol–water (100:40:3, v/v/v) (XIII) at a flow rate of 1 mL/min. For RP-HPLC analysis, a Nucleosil column (5-µm, 250- × 4.6-mm i.d.) (AB&L Jasco, Budapest, Hungary) eluted with a water–acetonitrile (77:23, v/v) (XIV) solvent system at a flow rate of 1 mL/min was used. Semipreparative RP-HPLC determinations were carried out on a Nucleosil (5 µm, 250- × 9.4-mm i.d ) (AB&L Jasco) column eluted with water–acetonitrile (77:23, v/v) (XIV) at a flow rate of 1 mL/min. Chromatographic separations were monitored at 242 nm.

### Droplet counter-current chromatography

An Eyela DCC-A instrument (Rikakikai, Tokyo, Japan) was used with chloroform–methanol–water (65:20:20, v/v/v) (XV) as the solvent system. The sample (0.9 g) was dissolved in 3 mL of the upper phase. A flow rate of 20 mL/h was applied, and 7-mL fractions were collected. The descending mode of operation was used, and the separation was repeated twice.

### Isolation procedure

Cleanup and prepurification consisted of extraction with methanol, filtration, fractionated precipitation (using acetone), liquid–liquid extraction with benzene, and SPE on alumina, as detailed in an earlier publication (6). The isolation scheme is given in Figure 1.

Fractions eluted with dichloromethane–methanol (4:1) from alumina (24.79 g dry residue) were used to isolate 20-hydroxyecdysone (19.3 g) by repeated crystallization in ethyl acetate–methanol (2:1). One part of the mother liquid (2.1 g dry residue) was fractionated by droplet counter-current column chromatography (DCCC) using Solvent System XV in descending mode. DCCC fractions 40–56 were dried, and the residue (1.3 g) was subjected to RP-LP-CC. Elution was carried out with a stepwise gradient of 30%, 35%, 40%, 45%, 50%, 55%, and 60% aqueous methanol (1800 mL each), and 300 mL of the fractions were collected. Fractions 30–42 (105 mg dry residue) were crystallized in ethyl acetate–methanol (2:1, v/v) to give compound 1 (50 mg). Ecdysteroids of the mother liquid (55 mg) were further purified by NP-HPLC (Solvent System XI) to yield four HPLC fractions: containing compounds 2 (7 mg), 3 (10.7 mg), 4 (1 mg), and 1 (31 mg), respectively. The final purification of 2 and 3 was performed by RP-HPLC (Solvent System XIV), preparing pure 2 (3 mg) and 3 (5 mg).

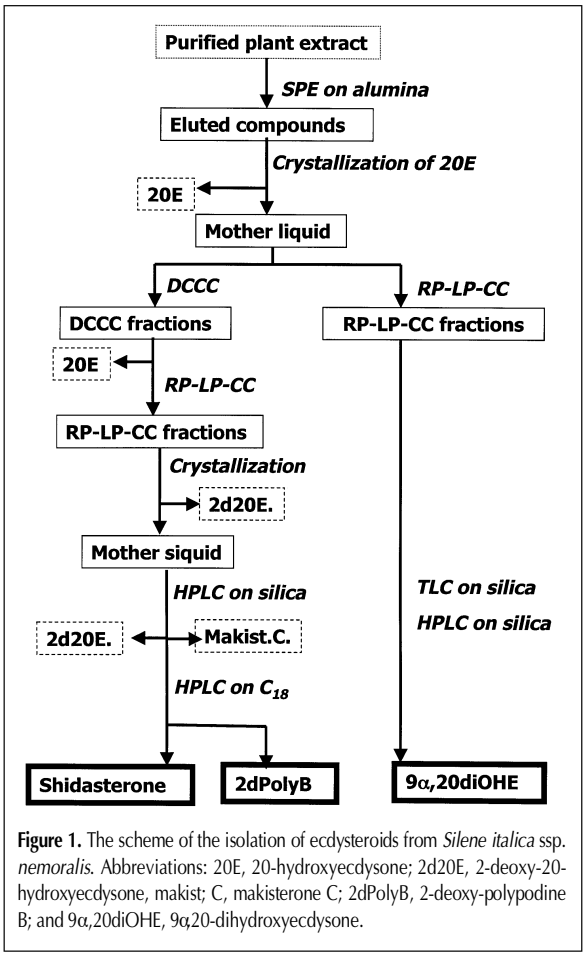



**Figure 1.** The scheme of the isolation of ecdysteroids from *Silene italica* ssp. *nemoralis*. Abbreviations: 20E, 20-hydroxyecdysone; 2d20E, 2-deoxy-20-hydroxyecdysone, makist; C, makisterone C; 2dPolyB, 2-deoxy-polypodine B; and 9α,20diOHE, 9α,20-dihydroxyecdysone.

The other part of the mother liquid of 20-hydroxyecdysone was again subjected to crystallization from ethyl acetate–methanol (3:1, v/v) to give pure 20-hydroxyecdysone (1.3 g). The mother liquid (1.9 g dry residue) was applied to RP-LP-CC and eluted with a 30–60% stepwise gradient of aqueous methanol (1800 mL each), and 300 mL of the fractions were collected.

The dry residue of fractions 6–10 (0.068 g) was subjected to preparative TLC (Solvent System III). This resulted in two major bands; one of them (6 mg) was further purified by NP- HPLC with Solvent System XI to prepare pure compound 5 (2 mg).

## Results

The minor ecdysteroids of interest were characterized on the basis of their retention behavior by HPLC. Figure 2 shows the NP-HPLC separation of the constituents of the prepurified methanol extract of *Silene italica* ssp. *nemoralis*. Almost every HPLC peak represents an ecdysteroid. 20-Hydroxyecdysone, the main ecdysteroid component of the extract, gave the highest chromatographic peak. The concentration of 20-hydroxyecdysone in the extract was approximately 10–1000-fold higher than that of the other ecdysteroids. The minor ecdysteroids of interest were present in approximately 0.001–0.004% of the gross dry residue of the raw extract.

### Cleanup and preliminary purification

The principal procedures were preparative scale separation methods, such as solid–liquid extraction and precipitation, as well as liquid–liquid extraction and SPE.

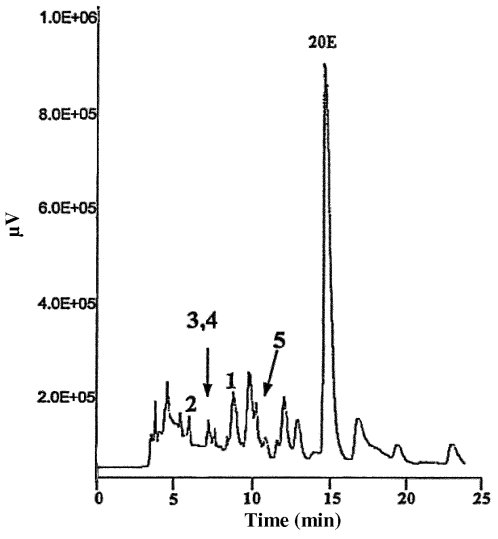



**Figure 2.** HPLC chromatogram of the prepurified extract of *Silene italica* ssp. *nemoralis* obtained by fractionated precipitation and liquid–liquid extraction of the crude extract. The stationary phase was a Zorbax-SIL column with dichloromethane–isopropanol–water (125:40:3) (solvent system XI), at a flow rate of 1 mL/min. The peaks represent compounds 1 (2-deoxy-20-hydroxyecdysone), 2 (shidasterone), 3 (2-deoxy-polypodine B), 4 (makisterone C), and 20E (20-hydroxyecdysone).

The proper solvent of solid–liquid extraction is methanol, extracting a large amount of both apolar and polar substances. To remove the nonecdysteroid type of compounds, both fractionated precipitation and liquid–liquid partition had to be used. Fractionated precipitation with acetone was found as an adequate process to remove the relatively polar contaminants, such as saccharides, proteins and salts.

The best choice for partition was benzene. The relatively hydrophilic ecdysteroids (having several hydroxyls) cannot be extracted by benzene, which removes the impurities, such as chlorophyll, plant pigments, terpenes, and sterols. The latest step in the prepurification was SPE on alumina. The ratio of sample to sorbent can be relatively large, up to 1:5. The polar impurities have a greater affinity to the stationary phase, therefore these impurities were retained on the alumina. The ecdysteroids can be eluted with the proper solvents, such as a mixture of a chlorinated hydrocarbon and alcohol. Here, a 4:1 ratio of dichloromethane and ethanol (96%) to elute the ecdysteroids of interest was used. Low water content of the solvent (such as 1%) decreases the adsorption of ecdysteroid on the stationary phase. However, the extract contains, even after prepurification, a complex mixture of structurally related compounds (mainly ecdysteroids), and their isolation requires chromatographic steps.

### Purification

SPE on alumina gave fractions enriched in 20-hydroxyecdysone and also several other minor components. The high excess of 20-hydroxyecdysone was removed by crystallization. DCCC was an effective method to remove the remaining part of 20-hydroxyecdysone. One half part of the crystallization mother liquid was subjected to DCCC. Selected DCCC fractions were purified by LC-RP-CC. An aqueous methanol mobile phase was used in the step-wise gradient elution, increasing the methanol content from 30% to 60%, with 5% in each step. The minor ecdysteroids were obtained at elution with high-methanol content. Fractions eluted with 55% and 60% aqueous methanol were

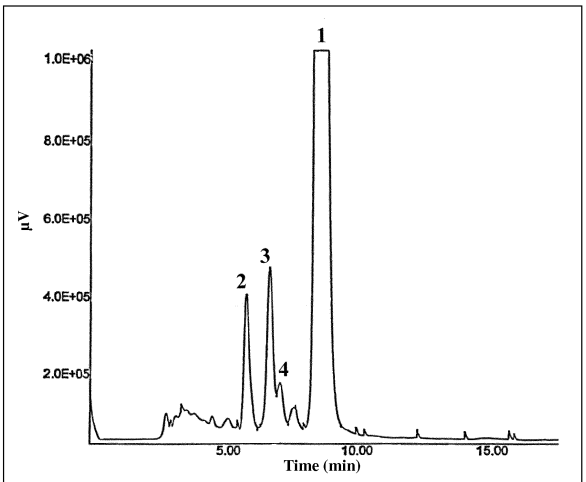



**Figure 3.** Analytical NP-HPLC separation of the fraction obtained by DCCC. NP-HPLC chromatography was carried out on a Zorbax-SIL column with dichloromethane–isopropanol–water (125:40:3) (solvent system XI) at a flow rate of 1 mL/min.

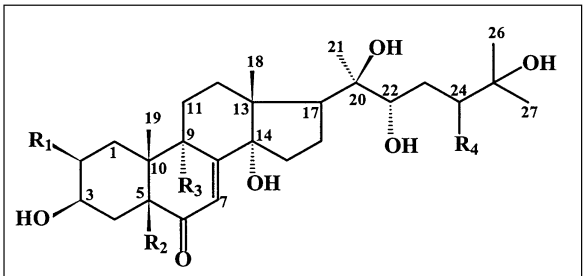
enriched in compound 1 (2-deoxy-20-hydroxyecdysone), but three other ecdysteroids were coeluted with compound 1 (2-deoxy-20-hydroxyecdysone) during the RP-LP-CC: compounds 2 (shidasterone), 3 (2-deoxy-polypodine B), and 4 (makisterone C).

The excess of compound 1 (2-deoxy-20-hydroxyecdysone) was also removed by crystallization. NP-HPLC was used for further separation. The result (Figure 3) indicates isolation of compounds 2 (shidasterone), 3 (2-deoxy-polypodine B), 4 (makisterone C), and 1 (2-deoxy-20-hydroxyecdysone). Compound 2 (shidasterone) and 1 (2-deoxy-20-hydroxyecdysone) gave well-separated peaks (the first and the last major peaks). However, the peak of 4 (makisterone C) was eluted as the shoulder following the peak of 3 (2-deoxy-polypodine B). Combining the fractions from the leading part of the peak of compound 3 (2-deoxy-polypodine B) gave the purified compound. Compound 4 (makisterone C) was obtained from processing the tailing of its peak. RP-HPLC separation yielded the ecdysteroids 2 and 3, pure enough for UV, NMR, and mass spectra.

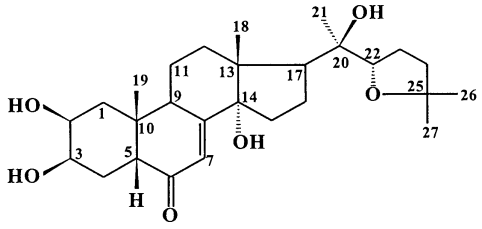
The other part of the mother liquid of 20-hydroxyecdysone was purified RP-LP-CC, and separation was completed using NP preparative TLC and NP-HPLC to yield pure compound 5 (9α,20-dihydroxyecdysone). The same procedure was used for the isolation of 9β,20-dihydroxyecdysone. Its isolation (using RP-LP-CC, TLC on silica, and HPLC on silica) will be reported later.

The structural elucidation of compounds 1, 2, 3, 4, and 5 were carried out using spectroscopic methods. Their chemical structures are given in Figure 4. Compounds 2 and 3 were proven to be shidasterone and 2-deoxy-polypodine B, and compound 5 was found to be 9α,20-dihydroxyecdysone. Compounds 1 and 4 were identified as 2-deoxy-20-hydroxyecdysone and makisterone C, respectively. Publication on the detailed evaluation of their spectral data is in progress.

Table I gives the TLC characteristics ($R_F$) of these compounds and of the reference 20-hydroxyecdysone. HPLC retention data of these ecdysteroids are listed in Table II.




| Compounds | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 20-Hydroxyecdysone (**20E**) | OH | H | H | H |
| 2-Deoxy-20E (**1**) | H | H | H | H |
| 2-Deoxy-polypodine B (**3**) | H | OH | H | H |
| Makisterone C (**4**) | OH | H | H | $C_2H_5$ |
| 9α,20-Dihydroxyecdysone (**5**) | OH | H | OH | H |




Shidasterone (**2**)

**Figure 4.** The chemical structures of the isolated ecdysteroids.

## Discussion

Our cleanup included several steps of simple separation methods. The large sample load required the use of preparative scale method (4). Our procedure for enriching the ecdysteroids from the extract was effective and inexpensive. The adequate selection of the organic solvents assured the proper selectivity. Both very polar and apolar impurities of the crude extract were removed with the successive use of fractionated precipitation and liquid–liquid extraction, followed by SPE. SPE on alumina offers a unique opportunity for a simple and relatively high throughput step of the ecdysteroid isolation (7). By NP-SPE, polar compounds are retained by the sorbent. Choosing a solvent series with increasing polarity will result in the sequential elution of the lead compounds, the ecdysteroids. Compounds out of our interest are retained on the stationary phase. The ecdysteroids were more moderately retained than the other constituents (sugars, amino acids, saponins) of the extract, and they can be selectively removed with a subsequent wash step. Capacity is a primary concern for such an application. The cleanup was followed by preparative scale chromatography

**Table I. TLC Relative Migration ($R_F$) of the Isolated Phytoecdysteroids***

| | $R_F \times 100$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | NP-TLC solvent system | | | | | RP-TLC solvent system | | | | |
| Compound | I. | II. | III. | IV. | V. | VI. | VII. | VIII. | IX. | X. |
| 2-Deoxy-20E (1) | 49 | 46 | 53 | 43 | 42 | 28 | 29 | 59 | 31 | 52 |
| Shidasterone (2) | 44 | 60 | 59 | 55 | n.d.† | n.d. | 17 | 67 | 18 | 34 |
| 2-Deoxy-polypodine B (3) | 46 | 56 | 58 | 60 | n.d. | n.d. | 28 | 61 | 28 | 47 |
| Makisterone C (4) | 41 | 46 | 55 | 50 | n.d. | n.d. | 28 | 60 | 30 | 41 |
| 9α,20-Dihydroxyecdysone (5) | 37 | 38 | 47 | 39 | n.d. | n.d. | 46 | 45 | 46 | 65 |
| 20-Hydroxyecdysone (20E) | 29 | 28 | 37 | 24 | 21 | 4 7 | 47 | 35 | 47 | 66 |

* Solvent systems: I, toluene–acetone–ethanol (96%)–ammonia (25%) (100:140:32:9, v/v/v/v); II, dichloromethane–methanol–benzene (25:5:3, v/v/v); III, ethyl acetate–ethanol (96%)–water (16:2:1, v/v/v); IV, dichloromethane–ethanol (96%) (8:2, v/v); V, ethyl acetate–methanol–ammonia (25%) (85:10:5, v/v/v); VI, acetonitrile–water with trifluoroacetic acid (0.1%) (25:75, v/v); VII, acetonitrile–water with trifluoroacetic acid (0.1%) (35:65, v/v), VIII, ethyl acetate–formic acid–water (85:10:5, v/v/v); IX, methanol–water (6:4, v/v); and X, tetrahydrofuran–water (45:55, v/v).
† n.d., not determined.

**Table II. HPLC Retention Times of the Isolated Phytoecdysteroids**

| | $t_R$(min) | | | |
|---|---|---|---|---|
| | NP* | | | RP† |
| | HPLC systems | | | |
| Compound | XI. | XII. | XIII. | XIV. |
| 2-Deoxy-20E (1) | 8.4 | 12.7 | 13.3 | 9.96 |
| Shidasterone (2) | 5.9 | 7.5 | 10.6 | 23.3 |
| 2-Deoxy-polypodine B (3) | 6.7 | 8.7 | 9.7 | 7.7 |
| Makisterone C (4) | 7.2 | 10.5 | 13 | 15.6 |
| 9α,20-Dihydroxyecdysone (5) | 10.5 | 16 | 18.7 | 6.45 |
| 20-Hydroxyecdysone (20E) | 15.3 | 23.8 | 22 | 4.2 |

* NP-HPLC was carried out on a Zorbax-SIL column (5 µm, 250- × 4.6-m i.d.) with the following solvent systems (flow rate 1 mL/min): XI, dichloromethane–isopropanol–water (125:40:3, v/v/v); XII, dichloromethane–isopropanol–water (125:30:2, v/v/v/v); and XIII, cyclohexane–isopropanol–water (100:40:3, v/v/v).

† RP-HPLC was carried out on an ODS-283 (6 µm, 250- × 4.6-mm i.d) with XIV, aqueous trifluoroacetic acid (0.1%)–acetonitrile (77:23, v/v) with a flow rate of 1 mL/min.

for the isolation of the pure analytes.

The isolation of 20-hydroxyecdysone is practically completed by its twofold crystallization, resulting in a yield over 80%. However, the remaining amount of 20-hydroxyecdysone may contaminate the chromatographic pattern of the minor ecdysteroids. Selection of DCCC or RP-LP-CC depends on the target compounds to be separated. DCCC provides more selective separation of the remaining part of 20-hydroxyecdysone from the minor ecdysteroids of interest than RP-LP-CC. However, when 9α,20-dihydroxyecdysone and 9β,20-dihydroxyecdysone (to be published later) having similar polarity to 20-hydroxyecdysone are targeted, RP-LP-CC is the optimal choice. When shidasterone and 2-deoxypolypodine B are to be isolated, DCCC offers better separation than RP-LP-CC. Both DCCC and RP-LP-CC can be suitably used to purify 2-deoxy-20-hydroxyecdysone and makisterone C.

It was possible to enrich certain minor ecdysteroids by using either DCCC or RP-LP-CC. These two methods have been used in parallel because an intent was to study the differences between their separation characteristics. DCCC separation of the ecdysteroids is based on the differences of their partition between the aqueous and organic phases. However, the absence of any sorbent permitted pure partition chromatography without any adsorption.

RP-LP-CC separation of ecdysteroids is based on their different lipophilicity and partition. However, neither DCCC nor RP-LP-CC alone has been efficient enough. In the present report, conditions were presented for these two methods in combination with TLC and HPLC to separate more efficiently the ecdysteroids.

The isolated ecdysteroids are minor constituents of the herb *Silene italica* ssp. *nemoralis*. Shidasterone, 2-deoxy-polypodine B, and 9α,20-dihydroxyecdysone are reported the first time from the *Silene* genus, and even in the Caryophyllaceae family. These latest two ecdysteroids are newly isolated from a natural source. 2-Deoxy-20-hydroxyecdysone and makisterone C are common ecdysteroids of the plant species (8).

## Conclusion

Ecdysteroids occur in the plants in complex mixtures, which also contain a high excess of interfering components. A proper selection of the isolation methods has to be combined for successful isolation of minor ecdysteroids. Moreover, adequate combination of the chromatographic methods should be sequenced to the purity of compounds suitable for structural elucidation.

## Acknowledgments

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