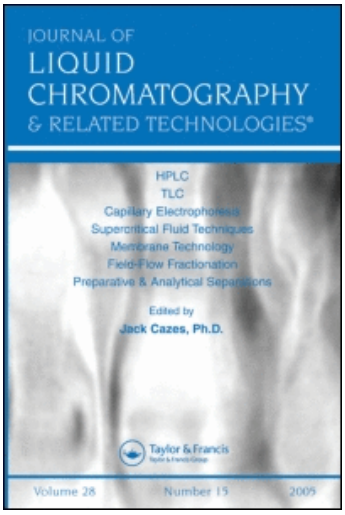


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 01 October 2000

**To cite this Article** Báthori, M. , Gergely, A. , Kalász, H. , Nagy, G. , Dobos, Á. and Máthé, I.(2000) 'LIQUID CHROMATOGRAPHIC MONITORING OF PHYTOECDYSTEROID PRODUCTION OF *SERRATULA WOLFFII*', Journal of Liquid Chromatography & Related Technologies, 23: 2, 281 — 294
**To link to this Article: DOI:** 10.1081/JLC-100101452
**URL:** http://dx.doi.org/10.1081/JLC-100101452

J. LIQ. CHROM. & REL. TECHNOL., 23(2), 281–294 (2000)

# LIQUID CHROMATOGRAPHIC MONITORING OF PHYTOECDYSTEROID PRODUCTION OF *SERRATULA WOLFFII*

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## ABSTRACT

Seasonal dependence of 20-hydroxyecdysone content of *Serratula wolffii* was monitored. Thin-layer chromatography was used after a single clean-up using polyamide. High performance liquid chromatography was employed for the analysis, after removal of the high excess of flavonoids and other impurities using a clean-up on both C18 and polyamide.

Parallel analyses using both thin layer chromatography with densitometry and high performance liquid chromatography gave similar results at the determination of vegetation dependence of ecdysteroids. Results indicated two maxima of ecdysteroid content (w/w), such as beginning of vegetation (in April and May) and during blossoming (in August).

The two ecdysteroids, 20-hydroxyecdysone and polypodine B were also isolated from *Serratula wolffii,* their chemical structures were identified by spectroscopic methods.

## INTRODUCTION

Ecdysteroids are widely spread in both insects and plants and they are characteristic steroids for more than 120 plant families. The occurrence of 20-hydroxyecdysone in plants in over 0.1% is not unusual. At the same time, prominently high ecdysteroid content (about or over 1%) has been hitherto detected only in 5 plant families. One of them is *Asteraceae* family. Especially, the 20-hydroxyecdysone content of *Serratula* species is high (between 0,9% and 2%).[1]

Biotests, radio immunoassay, HPLC, and also their combinations are used to determine ecdysteroid content of plant samples.[2-7] The same methods are also used to monitor the changes of the ecdysteroid content through the vegetation of plants.[6,7] At the same time, thin-layer chromatography combined with densitometry (TLC/DENS) has not been used, although TLC could be preferred for a series of determinations.



During our earlier work, we elaborated a TLC/DENS method to determine 20-hydroxyecdysone content of *Serratula tinctoria* L. (Wib).[8] With this method we followed quantitative changes of 20-hydroxyecdysone during vegetation. Our reason in this further work was, that:

(1) performing systematic investigations to determine 20-hydroxy-ecdysone of further *Serratula* species with the method,

(2) comparing the procedure to other analyses, first to HPLC. Thereby numerous data have been obtained for the applicability of the TLC/DENS.

Our work has been focused to the investigation of *Serratula* species, because determination of 20-hydroxyecdysone content of these interesting methodical problems are occurring:

(1) *Serratula* species synthesize significant amounts of polypodine B in addition to high 20-hydroxyecdysone concentration,[9-11]

(2) high amounts of phenoloics (flavonoids) are present that interfere with ecdysteroids.[12]

Polypodine B and 20-hydroxyecdysone show very similar chromato-graphic behaviour, their successful separation was done only with NP.[13] As the consequence of the high concentration of phenolic compounds in the *Serratula* species,

sample clean-up is necessary prior the determination. As flavonoids are the frequent metabolites of the plants, and polypodine B is the most frequently synthesized phytoecdysteroid after 20-hydroxyecdysone, by the solution of the above mentioned analytical problem, we reach a determination method that can be used at other numerous plant (*Spinacia oleracea*, *Chenopodium* species, *Leuzea carthamoides*).[12,14]

The occurrence of 20-hydroxyecdysone and polypodine B in plants has been verified by isolation, and chromatographic and spectroscopic identification. Moreover, CD spectra of two compounds are also given.

Our recent work deals with the study on the changes of ecdysteroids in the development of *Serratula wolffii*. Both TLC/densitometry and HPLC were used for the determinations, and the results gained by these two methods can also be compared.



## EXPERIMENTAL

### Plants

*Serratula wolffii* was cultivated from seeds, and collected in the Soroksár (vicinity of Budapest, Hungary) at the experimental fields of the Department of Medicinal Plant Cultivation of the University of Horticulture, Budapest, Hungary. The seeds of *Serratula wolffii* were received from Dijon in 1988 on the basis of an international agreement. Samples were collected in six different time points 1993, namely:

| | |
|---|---|
| April 28, | stage of rosette, |
| May 17, | beginning of stem formation, |
| June 18, | stem formation, beginning of shooting, |
| July 1, | bud formation, bud development, |
| August 8, | blossoming, |
| September 5, | ripening |

### Standard Compounds

20-Hydroxyecdysone and polypodine B (5,20-dihydroxyecdysone) were originated from our earlier preparative work.[9,10] Figure 1. presents the chemical structure of 20-hydroxyecdysone and that of polypodine B.

Solvents and chemicals were purchased from Reanal Fine Chemical Works (Budapest, Hungary). Silica for column chromatography (0.063-0.2 mm) and TLC plates 20 x 20 cm, silica gel 60 $F_{254}$ were purchased from E. Merck (Darmstadt, Germany), while alumina Brockmann II, neutral was bought from

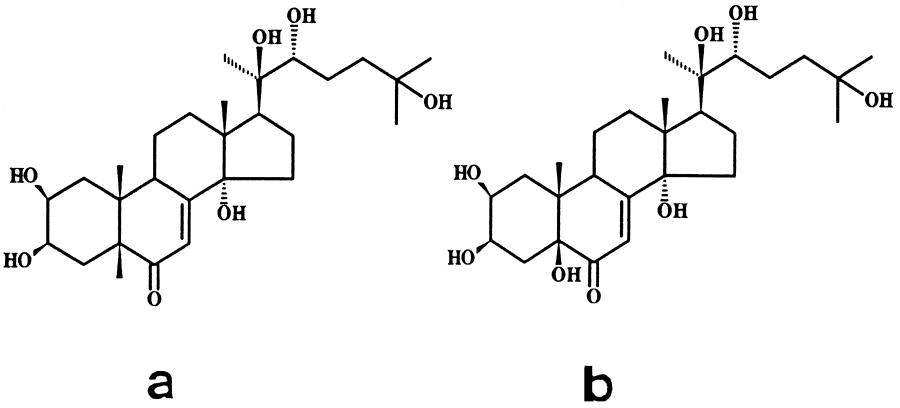


**Figure 1**. a) The chemical structure of 20-hydroxyecdysone; b) The chemical structure of polypodine B.



Reanal (Budapest, Hungary). For sample clean-up MN Polyamide DC6 for TLC (0.005-0.16 mm, Macherey-Nagel, Düren, Germany) and Samplex C18 (octadecyl silica, particle size: 40 μm, BioSeparation Techniques, Budapest, Hungary), 2 mL/250 mg each, were used.

## Instrumentation

Thin-layer chromatography was done with a Camag UV lamp at 254 nm was used for visualisation of spots. Quantitative determinations of 20-hydroxyecdysone from samples used Shimadzu C9301 PC Scanner (Shimadzu Ltd., Tokyo, Japan) at 254 nm with reflectance mode. TLC silica plates $F_{254}$ were used. Mobile phases were:

| | |
|---|---|
| dichloromethane-ethanol (96%) | (8:2, v/v), |
| chloroform-methanol-benzene | (25:5:2, v/v/v), |
| ethyl acetate-methanol-concentrated ammonia | (85:10:5, v/v/v). |

Detection of ecdysteroids and contaminants were done directly after development under UV light at 254 nm, and also after using vanillin-sulphuric acid spray reagents, and observing the spots both in daylight and under UV light.

HPLC experiments were performed using Shimadzu LC-10AS Pump, SPD-10A Shimadzu UV-VIS Detector, and Hypersil (6 μm) stationary phase packed in a 4.6 mm x 250 mm stainless steel column, supplied by BST Company

(Budapest, Hungary). HPLC experiments were performed at ambient temperature. The mobile phase was dichloromethane-isopropanol-water (125:40:3, v/v) with flow rate of 1 ml/min; 20 μL samples were injected. Detection was done at 254 nm, AUFS was 0.01.

Circular dichroism of the pure 20-hydroxyecdysone and polypodine B was determined using J-720 type Spectropolarimeter of Jasco (Japan Spectroscopic Co., Ltd, Tokyo, Japan).

**Isolation of 20-Hydroxyecdysone and Polypodine B**

Prepurification started with drying and milling the herba of *Serratula wolffii*. The powdered plants were swollen with methanol (2 kg in 10 liter) for 10 hours and percolated. Pre-purification of the crude extract was proceeded as detailed in our earlier work.[9] The crude extract was taken into dryness in a rotatory evaporator under 4 Hgmm of vacuum, the temperature of the water bath was 40°C. The residue (232.9 g) was dissolved in 1250 mL methanol. mixed with 600 ml acetone, and the precipitate was filtered out through a G3 glass filter (Pyrex, France), washed three times with 30 mL of a 1:2 (v/v) mixture of acetone-methanol.

The filtrate and the washing solutions were combined and evaporated using a rotary evaporator and the conditions mentioned above. The 166.9 g dry residue was dissolved in 700 mL methanol, mixed with 700 mL acetone, and the precipitate was filtered through a G3 glass filter. The precipitate was washed three times with 30 mL of a 1:1 (v/v) mixture of acetone-methanol. The washing solution and the filtrate were combined, and taken into dryness using a rotary evaporator and the conditions mentioned above. The dry residue (124.5 g) was dissolved in 500 mL 50% aqueous methanol. The solution was extracted four times with 1000 mL benzene, the upper (benzene) phase was discarded.

The aqueous methanol phase was evaporated using a rotary evaporator and the conditions mentioned above. The 108 g residue was dissolved in 300 mL methanol, and mixed with and adsorbed on silica (200 g) that was layered on the top of a 1000 g silica (packed in a glass column having 100 cm length and 5.5 cm inner diameter).

Ecdysteroids were eluted using stepwise gradient starting from dichloromethane and completing with dichloromethane-ethanol (96%) 8:2 ratio. Individual steps of the gradient were 4.8 liter dichloromethane, 8 liter dichloromethane-ethanol (96%) 95:5 (v/v), 8.8 liter dichloromethane-ethanol (96%) 9:1 (v/v), 7.2 liter dichloromethane-ethanol (96%) 85:15 (v/v), and 1.6 liter dichloromethane-ethanol (96%) 8:2 (v/v). The flow rate was about 10 mL/min. Fractions were collected, 800 mL each. Having collected 10 fractions in one day, the elution was stopped and continued on the other day.

Each step of the elution was monitored by the help of TLC analysis using solvent systems Nos. 1. and 3. Fractions through 27-30 (eluted with dichloromethane-ethanol (96%) 9:1 and also 85:15 resulting in polypodine B), and fractions through 31 and 35 (eluted with dichloromethane-ethanol (96%) 85:15 and resulting in 20-hydroxyecdysone) were combined and subjected to further purification.

Fractions 27-30 were evaporated, dissolved in methanol (4.3 g in 100 mL), and adsorbed on polyamide (9 g). A column was packed (9 cm x 2.5 cm, i.d.), and eluted with water (200 mL in 120 min). Polypodine B containing fractions (3.5 g, dry weight) were combined, mixed with 10 g silica, taken into dryness, suspended in ethyl acetate-methanol-concentrated ammonia solution (80:10:5, v/v/v), and layered on a column of 2.5 x 6.5 cm, i.d.. The column was eluted with the same mobile phase as used for suspension; the flow rate was 1 mL/min. Fractions were collected, 25 ml, each.



Fractions through Nos. 3 and 6 were combined, placed and into refrigerator; crystalline polypodine B was obtained. Pure polypodine B was gained by recrystallization from methanol. Crystalline polypodine B showed a melting point at 254-257°C, the same as given in the literature.[15] The chemical structure of polypodine B was verified by spectroscopic methods.

Fractions through 31-35 of chromatography on 1000 g silica column were combined, evaporated, and the residue was dissolved in methanol (13.6 g in 100 mL). After adsorption on 68 g alumina it was layered on the top of a column of 100 x 3 cm, i.d., packed with 400 g alumina, and eluted with 15 liter chloroform-ethanol (96%) (9:1, v/v) with 5 mL/min. Fractions were collected, 100 mL each.

Fractions through 35-145 (containing 20-hydroxyecdysone) were combined, kept refrigerated, and yielded crystalline 20-hydroxyecdysone in 24 hours. Crystals were filtered, washed with ethyl acetate, and recrystallized from ethyl acetate-methanol (2:1, v/v). Melting point of the crystals was 241-242°C, corresponding to the literature.[15] Chemical structure of 20-hydroxyecdysone was verified by spectroscopic methods.[9]

## TLC/Densitometry Determination of 20-Hydroxyecdysone

TLC/densitometry determination of 20-hydroxyecdysone from various phenophases of *Serratula wolffii* was performed. Plant samples (leaf, stem) were collected in the various phenophases (detailed previously), dried at room temperature, and milled. Aliquots of 0.5 g from each sample were taken, extracted three times with 5 mL methanol for 15 sec. by the help of an ultrasonic bath and the extracts of each sample were filtered through the same filter paper. The filter paper was also washed with 3 x 5 mL methanol; filtrate and washing solutions were combined, mixed with 0.5 g polyamide.

The suspension of methanol and polyamide were dried using a rotator evaporator from a water bath (40°C), the vacuum (4 mm Hg) was done by a water-jet pump. The residue was transferred to a glass filter, and ecdysteroids were eluted with 2 x 5 mL water in 1 min. The filtrate/eluent aqueous solution was completed to 10 mL, and 20 µL aliquots were spotted on TLC plates. Plates were twice developed (first in TLC system No. 1, then in TLC system No. 2.). After the 1st development, the plate was dried and the 2nd development was performed in the same direction. The spots were scanned by densitometry.[8]

Evaluation of TLC/densitogram was done on the basis of calibration using 2, 5, 10, 30, 50, 70 µL of the test solution of 20-hydroxyecdysone (0.058 mg/mL methanol solution), and performing two parallel determinations. Calibration was done using a second order equation of the values of standards, and the numerical 20-hydroxyecdysone values were calculated using Albacomp Gold Start (Székesfehérvár, Hungary) computer and Microsoft Excel 5.0 program.



Correlation coefficient of the standard curve was 0.9992 through 1. Statistical evaluations, such as calculation of standard deviation, standard error, and 95% confidence limit were done using the statistical program of Excel 5.0 software.

**HPLC Determination of 20-Hydroxyecdysone**

Aliquot dried extracts corresponding to 0.5 g plant (leaf) were sonicated with 2 x 5 mL distilled water and filtered through a G3 glass filter. Samplex C18 columns were washed with 10 mL methanol, and activated by washing with 10 mL distilled water. Five milliliter filtrate was passed through the Samplex C18, washed with 5 mL 15% aqueous methanol. Washing solution does not elute ecdysteroid at all. Ecdysteroids were eluted with 5 mL 60% methanol. Ecdysteroid content of the effluent was monitored by HPLC. Effluent with 60% was evaporated using a rotary evaporator under 4 Hgmm of vacuum, the temperature of the water bath was 40°C. The residue was dissolved in 2 mL distilled water.

The solution was mixed with polyamide (0.2 gram), placed into a G3 glass filter (7.5 mm x 10 mm, i.d.), filtered, and also washed further with 2 mL water. The filtrate and the washing solution were combined, completed to 5 mL, and 20 µL aliquots were subjected to HPLC analysis. Further elution of polyamide did not result in ecdysteroids, however, analysis of methanolic elution of polyamide was solely done to clarify the absent of ecdysteroids and the presence of flavonoids.

Quantitative evaluation was done using calibration curve of 0.2, 0.15, 0.1, 0.06, 0.04 and 0.02 mg 20-hydroxyecdysone. Correlation coefficients of the standard curves were between 0.9993 and 0.9998.

**Table 1**

**20-Hydroxyecdysone Content of the Dried Leaves of *Serratula Wolffii**

| Month in 1993 | % Determined (Average) | Standard Deviation | Standard Deviation | 95% Confidency Limit |
|---|---|---|---|---|
| April | 0.8488 | ± 0.0685 | 0.0242 | 0.0573 |
| May | 0.8379 | ± 0.0731 | 0.0258 | 0.0611 |
| June | 0.1843 | ± 0.0165 | 0.0058 | 0.0138 |
| July | 0.3801 | ± 0.0519 | 0.0183 | 0.0434 |
| August | 0.4069 | ± 0.0645 | 0.0228 | 0.0539 |
| September | 0.1316 | ± 0.0176 | 0.0062 | 0.0147 |

* Standard deviation, standard error, and 95% confidency limit of results.



**Table 2**

**20-Hydroxyecdysone Content of the Dried Stem of *Serratula Wolffii**

| Month in 1993 | % Determined (Average) | Standard Deviation | Standard Deviation | 95% Confidency Limit |
|---|---|---|---|---|
| May | 0.3461 | ± 0.0330 | 0.0165 | 0.0526 |
| June | 0.0373 | ± 0.0093 | 0.0046 | 0.0147 |
| July | 0.0492 | ± 0.0049 | 0.0025 | 0.0079 |
| August | 0.0916 | ± 0.0155 | 0.0077 | 0.0247 |
| September | 0.0243 | ± 0.0074 | 0.0037 | 0.0118 |

* Standard deviation, standard error, and 95% confidency limit of results.

## RESULTS

Table 1 gives 20-hydroxyecdysone content of leaves of *Serratula wolffii,* determined in various phenophases (time points) with 8 independent TLC/densitometry determinations. Using Student's t-test, no significant differences were found between the values found in April and May, and July and August. Otherwise. the significance level is $p < 0.01$. Table 2 gives the 20-hydroxyecdysone content of the stem using TLC/densitometry; the averages of four independent determinations are given. There are no significant differences between the 20-hydroxyecdysone content in June and July and in June and September. Between all the other results, there is a significance level at $p < 0.01$.

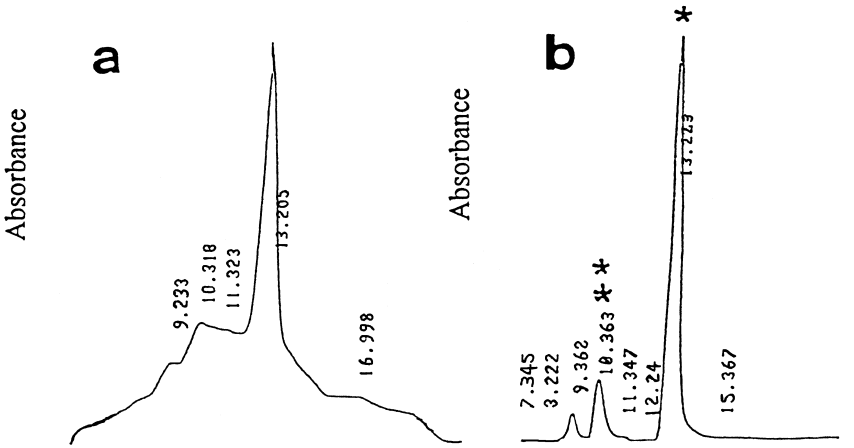


**Figure 2**. a) HPLC of methanolic extract of sample of August, 1993, followed the clean-up using Samplex C18. The Arabic numbers indicate retention time of peaks; b) HPLC of methanolic extract of sample of August, 1993, involved both the clean-up using Samplex C18 and small polyamide column. The Arabic numbers indicate retention times of peaks.



Figure 2a gives a HPLC chromatogram obtained after a Samplex C18 clean-up. A combined clean-up of Samplex C18 followed by polyamide clean-up results in the decrease of contaminants as shown in Figure 2b. Figure 3 shows a comparison of 20-hydroxyecdysone content of leaves determined using HPLC to that of TLC.

Figures 4a and 4b show CD spectra of 20-hydroxyecdysone and polypodine B. Both substances have positive Cotton effect (CE) in the n —> $\pi^*$ transition. Molar ellipticity ([$\theta$]) of 20-hydroxyecdysone is 5697 at 330 nm and that of polypodine B is 9757 at 329 nm. Negative CE can be observed between 254 nm and 256 nm corresponding to n —> $\pi^*$ transition in the UV spectrum; [$\theta$]: (-) 12883 (20-hydroxyecdysone) and (-) 15930 (polypodine B.

Positive CE can be seen between 224 and 227 nm, which has no corresponding UV maximum; [$\theta$]: 10047 (20-hydroxyecdysone) and 22830 (polypodine B).

Optical rotation dispersion (ORD) curves have positive Cotton effect; molar rotation of 20-hydroxyecdysone is 2564 ($\lambda_{max}$: 357 nm), and that of polypodine B is 6019 ($\lambda_{max}$: 354 nm).

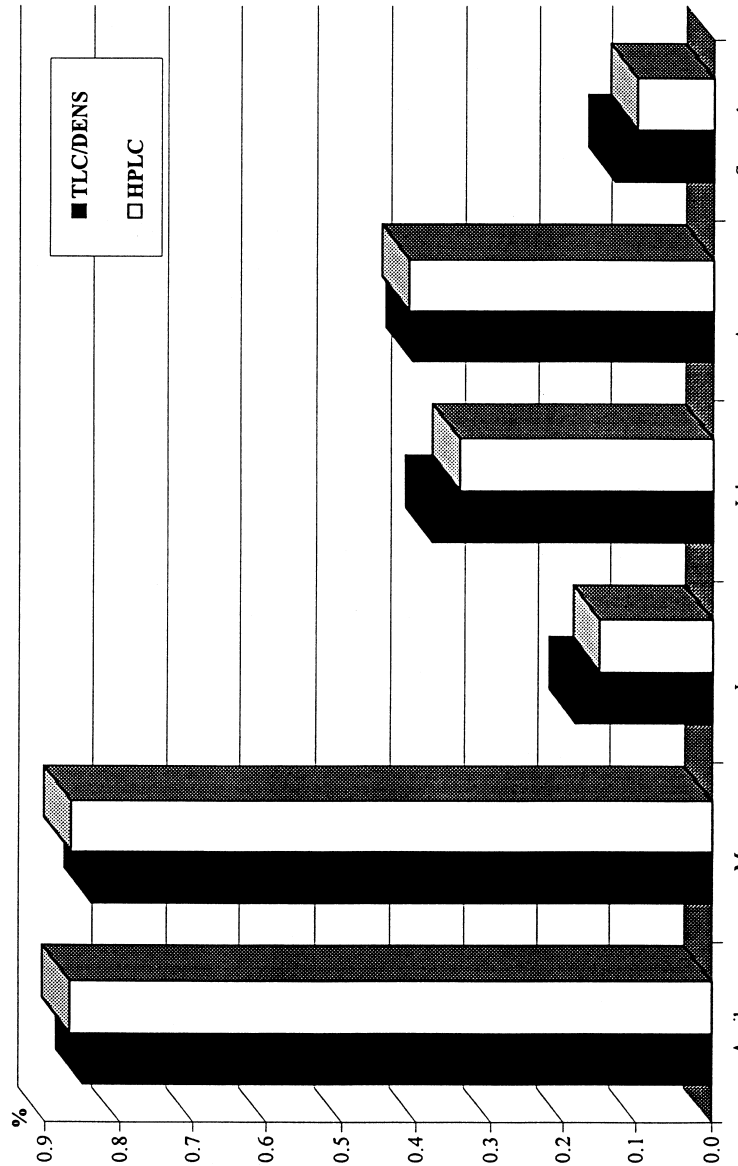


**Figure 3.** Change in 20-hydroxyecdysone content determined by HPLC and TLC/DENS of methanolic extract followed the clean-up.

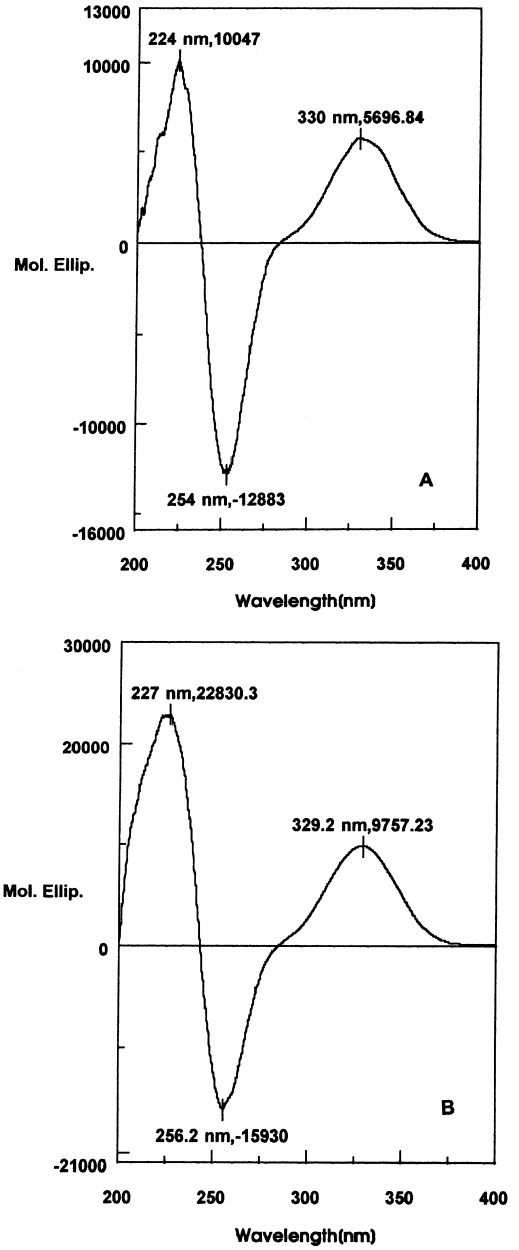


**Figure 4**. a) CD spectrum of 20-hydroxyecdysone in ethanolic solution; b) CD spectrum of polypodine B in ethanolic solution.

## DISCUSSION

Identifications of the isolated 20-hydroxyecdysone and polypodine B from *Serratula wolffii* were verified by HPLC and TLC characteristics, as well as spectroscopic data. The data correspond to that in the literature. Specific rotation and CRD may give additional data to the earlier spectroscopic methods such as mass spectrometry and nuclear magnetic resonance spectroscopy.[12]

Ecdysteroid content of plant samples was generally determined by the use of either radio immunoassay (RIA),[2,4] or spectrophotometry,[5] or thin-layer chromatography[8] or HPLC.[3,6,7] Either a clean-up or preliminary purification was generally applied.[2-8]

To determine 20-hydroxyecdysone content of *Serratula wolffii*, several points of view were taken into consideration, such as:

1. The method had to perform a series of determination by a simple and quick way.

2. A reliable chromatographic method was preferred.

3. To differentiate 5beta-OH and 5beta-H, substituted compounds (that is polypodine B and 20-hydroxyecdysone) by straight-phase chroma-tography was preferred to the reverse-phase methods.

4. In contrary to the other ecdysteroid containing plants, *Serratula wolffii* consists of a high abundance of flavonoids (> 1%), and flavonoids can interfere chromatographic determinations of ecdysteroids (Fig. 2a).

As the methods of choice include two straight-phase chromatographic procedure, TLC on silica plates and HPLC using silica column, both procedures were applied.

TLC/DENS does not generally require prepurification; the high amount of flavonoids present in *Serratula wolffii* interfere with the determination of 20-hydroxyecdysone. Therefore, to remove these phenoloids, prepurification should be performed. The sample preparation, which utilizes the differences in the absorption/elution of ecdysteroids and flavonoids on polyamide, is simple, fast, and effective[8] and does not essentially increase the time of determination.

Prior HPLC determination, application of solid phase extraction on Samplex C18, is not enough (Figure 2a). Filtration on polyamide layer has to be included that helps to obtain single peaks of ecdysteroids without interference with that of flavonoids (Figure 2b). As the prepurification is growing longer, and the fact that NP-HPLC has to be used for separation of polypodine B and 20-hydroxyecdysone, this will greatly increase the time requirement of analysis (equilibri-

um is reached slowly on NP). TLC/DENS also requires pre-purification, however, even a single step removal on polyamide makes the samples clean enough for TLC separation.[8] Therefore, we judge TLC/DENS more suitable if the sample contain large excess of flavonoids together with 20-hydroxyecdysone and polypodine B. The results of TLC and HPLC were in good agreement (Figure 3).

Maxima of 20-hydroxyecdysone content of leaves were found at two different stages of vegetation. At the beginning of annual development of the plants, in April and May, high 20-hydroxyecdysone content characterized the plant (almost 0.9%) determined by either TLC/densitometry and HPLC/ photometry. A declining period was ended in a minimum at June giving one fourth of the maximum. A rise of 20-hydroxyecdysone content gave another high 20-hydroxyecdysone content of the leaves at about 0.4%, however, the parallel increase of leave production resulted in a favorable period for harvesting in August. This period is marked by the blossoming of *Serratula wolffii*. After blossoming, 20-hydroxyecdysone content repeatedly decreased. The vegetation dependence of 20-hydroxyecdysone in the stem of *Serratula wolffi*i shows a similar tendency than that of the leaves. CD spectra of the isolated ecdysteroids supply a valuable information that either the homogenous spot of TLC analysis or the single peak of HPLC really represents the ecdysteroid to be determined.[16]



## ACKNOWLEDGMENT

This work was financially supported by a T 022,449 grant of OTKA, Budapest, Hungary.

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Received July 28, 1998
Accepted June 26, 1999
Manuscript 4841