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Efficient determination of phytoecdysteroids from *Ajuga* species and *Polypodium vulgare* by high-performance liquid chromatography

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ABSTRACT

Efficient chromatographic conditions were established for the simultaneous determination of eight phytoecdysteroids by high-performance liquid chromatography. Spherisorb ODS-2 columns, ultraviolet detection, isopropanol–water as the mobile phase and temperature control were used. Ecdysteroids were obtained by purification of methanol plant extracts with Sep-Pak C_{18} cartridges. The results compared well with those obtained by other chromatographic methods in terms of resolution, selectivity and efficiency.

INTRODUCTION

Ecdysteroids are a group of steroidal compounds related to the insect moulting hormones. They all have the same rigid 5β-cholestane skeleton with a 7-en-6-one chromophore and a variable number of hydroxyl substituents.

Several methods have been used for the determination of ecdysteroids[1–3]. High-performance liquid chromatography (HPLC) with or without prior derivatization was found to be quicker and easier to use than gas chromatography (GC) although GC with electron-capture detection exhibits a higher selectivity and sensitivity[4]. In addition, HPLC is a non-destructive method and fractions of pure compounds may be collected for identification or bioassay purposes[5].

Baseline resolution of many ecdysteroids can be achieved by HPLC on columns of microparticulate material[6]. Both silica and reversed-phase supports can be efficiently used but the solvent systems have to be optimized. Reversed-phase packings are the most commonly employed and selective effects of different mobile phases have been described[1]. Mixtures of methanol–water and acetonitrile–water with other organic modifiers such as tetrahydrofuran are the most common solvent systems reported. Acid buffers[7,8], cationic detergents[9] and high-performance columns (with a length of 20 or 25 cm) must be used for difficult separations, but these conditions

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usually cause baseline disturbances or lead to long time analysis. Moreover, the inorganic modifiers make work under preparative conditions difficult.

Plants contain ecdysteroids in larger amount than insects. More than 100 ecdysteroids have so far been isolated from plants[10,11], but only some of these compounds have also been detected in insects. During the course of our work on the production of ecdysteroids by plant tissue cultures from different *Ajuga* species and the fern *Polypodium vulgare*, we needed a suitable and efficient chromatographic method to determine these compounds using a peak-area standard. Owing to the number of quantitative analyses required, an important priority in this reasearch was the development of a reliable and effective HPLC separation method for the main ecdysteroids produced by these plants.

In this paper, we report an efficient and rapid chromatographic system for the simultaneous determination of eight different ecdysteroids present in *Polypodium vulgare* and several *Ajuga* species.

EXPERIMENTAL

Chemicals and materials

29-Norsengosterone (NS), 29-norcyasterone (NC). cyasterone (CY) and ajugalactone (AJL) standards were extracted from *Ajuga reptans*, 20-hydroxyecdysone (20-HE), ecdysone (E), 20,26-dihydroxyecdysone (20,26-HE) and polypodine B (PB) were obtained by purification from *Polypodium vulgare* rhizome extracts and makisterone-A (MK) was obtained from *Ajuga chamaepitys*. For the extraction and purification we used procedures already described for the isolation of ecdysteroids from biological samples[12,13], with slight modifications.

High-purity solvents (HPLC grade) were purchased from Merck, Scharlau or Romil. Water for HPLC was purified with a Milli-Q system and, prior to use, filtered through 0.45-μm Millipore filters (Type HA, for aqueous solvents) and degassed in ultrasonic bath under vacuum.

HPLC

Two Waters Assoc. Model 150 pumps were used for solvent delivery, controlled by a Waters Assoc. Model 660 solvent-programming unit. Samples were introduced via a Rheodyne 7105 injector, and compounds were detected with a Waters Assoc. Model 450 variable-wavelength UV spectrophotometer set at 242 nm. Temperature was regulated with a Perkin-Elmer Model 1220 oven. Chromatograms were recorded with a Waters Assoc. Model 740 data module integrator.

The analyses were carried out on a 25 × 0.4 cm I.D. (10 μm particle size) and 10 × 0.4 cm I.D. (5 μm) Spherisorb ODS-2 C_{18} (Tracer Analítica), 12 × 0.4 cm I.D. (3 μm) Spherisorb ODS-2 C_{18} (Scharlau) ad LiChroCART 12.5 × 0.4 cm I.D. (5 μm) LiChrospher 100 RP-18 (Merck) reversed-phase columns under isocratic conditions.

Preparation of samples

Different samples of *Ajuga* species (400–1000 mg) and *Polypodium vulgare* (25–50 mg) were extracted with methanol in an ultrasonic bath or homogenized with methanol and methanol–water mixtures. The methanolic extracts were centrifuged, washed with hexane and, after partial evaporation of the organic solvent, diluted with

water to 10 ml of *ca.* 10% aqueous methanol and retained on a reversed phase Sep-Pak C_{18} cartridge. Isolation of ecdysteroid fractions was carried out by elution of more polar substances than ecdysteroids with 10 ml of 15% aqueous methanol. Ecdysteroids were then eluted with 5.5 ml of 85% aqueous methanol, the first 0.5 ml were discarded and the next 5 ml were used for HPLC analysis. Less polar substances than ajugalactone in the case of *Ajuga* or ecdysone for *Polypodium* were not eluted under these conditions.

The ecdysteroid eluate from the Sep-Pak C_{18} cartridge was injected directly into the HPLC column after mixing with a solution of methyl anthranilate as a peak-area standard for quantitative analysis.

Quantification

Ecdysteroid concentrations were calculated by interpolation on the calibration graph obtained for 20-hydroxyecdysone as a representative of the more polar (first group) phytoecdysteroids and ajugalactone as a representative of the less polar compounds (last group in this present study) with methyl anthranilate (MA) as a peak-area standard. MA elutes between the two groups and does not interfere under

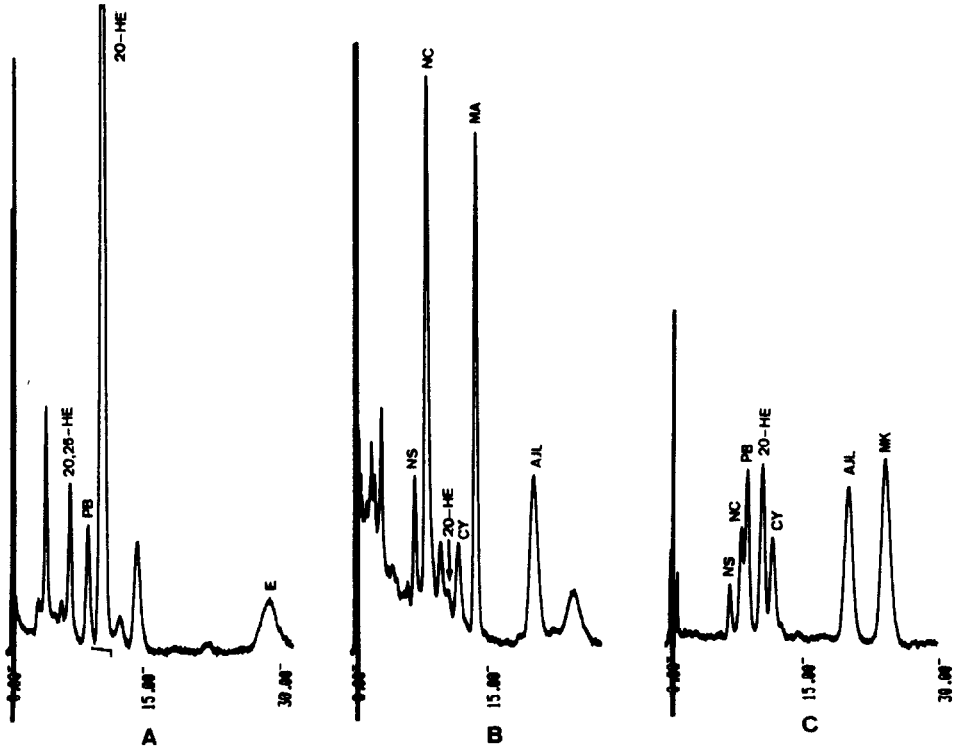

Fig. 1. Chromatographic profiles of plant extracts: (A) *in vitro* growing prothalli of *Polypodium vulgare*; (B) *in vitro* micropropagated plantlets of *Ajuga reptans*; (C) a mixture of standards. Column, LiChrospher 100 RP-18 (5 μm), 12.5 × 0.4 cm I.D., thermostated at 55°C; mobile phase, isopropanol–water (6.5:93.5) at 2 ml/min.

the conditions used. The result obtained was multiplied by a correction factor corresponding to the different absorptivities, the most important element affecting the area of an eluted product in comparison with other factors such as reversible and irreversible absorption on the column.

RESULTS AND DISCUSSION

The separation of polypodine B and 20-hydroxyecdysone, two of the ecdysteroids present in these plants, is regarded as a difficult problem in HPLC analysis[14], even with extended analysis time. However, as will be seen later, we have achieved this separation using isopropanol–water mixtures and a reversed-phase microparticulate support. A comparative study of different eluent systems and temperature conditions allowed us to determine the ecdysteroid content in methanolic extracts of *P. vulgare* and *Ajuga* species. Fig. 1 shows the chromatograms for two methanolic plant extracts.

The aim of all chromatographic analyses is to optimize the resolution in the minimum time. Resolution (R) is a function of the selectivity (α), the number of theoretical plates or efficiency (N) and the capacity factor (k').

The choice of an appropriate solvent system allows the optimization of α and k'. Mixtures of isopropanol and water were tested as eluents and compared with two normal solvent systems used in the chromatography of ecdysteroids, namely methanol–water and acetonitrile–water. A primary consideration was the need to find an isocratic system, if possible, without a buffer in the eluent, in order to be able to apply it for preparative purposes.

The mobile phase was optimized in order to separate 20-hydroecdysone and polpypodine B using the 10-μm column. The three systems were tested with different proportions of organic solvent, but the resolution was always poor when using acetonitrile–water mixtures even with prolonged analysis. The best results were obtained with isopropanol as organic modifier because it showed a higher resolution for a similar capacity factor.

Next, six other ecdysteroids, 29-norsengosterone, 29-norcyasterone, cyasterone, ajugalactone, ecdysone and makisterone-A, were tested, again comparing the three solvent mixtures. In this instance we employed the 5-μm column at room temperature. The best conditions were the use of methanol–water (30:70) or isopropanol–water (7:93), but the latter mixture exhibited a higher resolution. However, in no instance were all peaks completely separated down to the baseline. Ternary mixtures described in the literature, such as methanol–acetonitrile–water[5], were tried but not all the standards were separated under these conditions.

The relative retention times result from the different distribution coefficients of the components of the sample between the eluent and the stationary phase. With some exceptions (*e.g.*, exclusion chromatography) these distribution coefficients are thermodynamic parameters and, in consequence, they depend on the temperature, among other factors. This effect was studied by thermostating the column at various temperatures, employing two different systems and conditions. In the first instance the 5-μm column was used, the mobile phase was isopropanol–water (7:93) at a flow-rate 1 ml/min and the temperature was varied from 35 to 60°C. In the second, a reversed-phase 3-μm column was used, the mobile phase, was isopropanol–water (9:91) at a flow-rate of 0.9 ml/min and the temperature was varied from 23 to 63°C.

TABLE I

EQUATIONS OF ADJUSTED CURVES FOR THE VARIATION OF THE CAPACITY FACTOR WITH TEMPERATURE (T)

Stationary phase, Spherisorb ODS-2, 5 μm; column, 10 × 0.4 cm I.D.; mobile phase, isopropanol–water (7:93) at 1 ml/min. $T_{\frac{1}{2}}$ = exponential decay rate (°C).

Phytoecdysteroid	*Equation of exponential decay*	$T_{\frac{1}{2}}$	r
29-Norsengosterone (NS)	$k' = 21.6e^{-0.045T} + 3.0$	15.20	0.999
29-Norcyasterone (NC)	$k' = 32.0e^{-0.049T} + 3.3$	14.07	0.999
Polypodine B (PB)	$k' = 19.3e^{-0.038T} + 3.7$	17.87	0.998
20-Hydroxyecdysone (20-HE)	$k' = 29.1e^{-0.042T} + 4.1$	16.20	0.999
Cyasterone (CY)	$k' = 39.0e^{-0.046T} + 4.8$	15.06	0.999
Makisterone (MK)	$k' = 75.7e^{-0.044T} + 7.1$	15.60	1
Ajugalactone (AJL)	$k' = 76.8e^{-0.044T} + 8.6$	15.61	1

The same behaviour was observed in both instances. The capacity factor decreased with increase in temperature, following an exponential decay curve. The adjusted curves had similar exponents except for 29-norcyasterone and polypodine B (Table I). The former had a higher exponent and its retention time decayed faster than the others, whereas the exponent for polypodine B was lower and its retention time diminished at slower rate (Fig. 2). Consequently, both curves crossed at *ca.* 40°C, after which the elution of these two compounds was reversed.

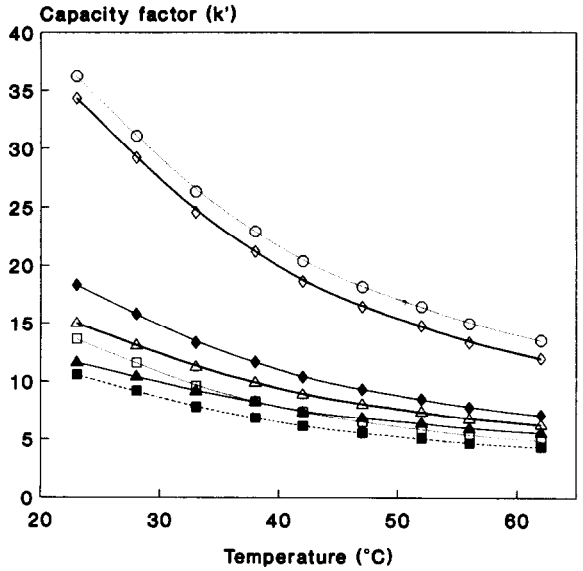

Fig. 2. Effect of temperature on the capacity factor. Column, 12 × 0.4 cm I.D. packed with Spherisorb ODS-2 (3 μm); mobile phase, isopropanol–water (9:91) at 0.9 ml/min. □ = NC; ▲ = PB; ■ = NS; △ = 20-HE; ◆ = CY; ◇ = MK; ○ = AJL.

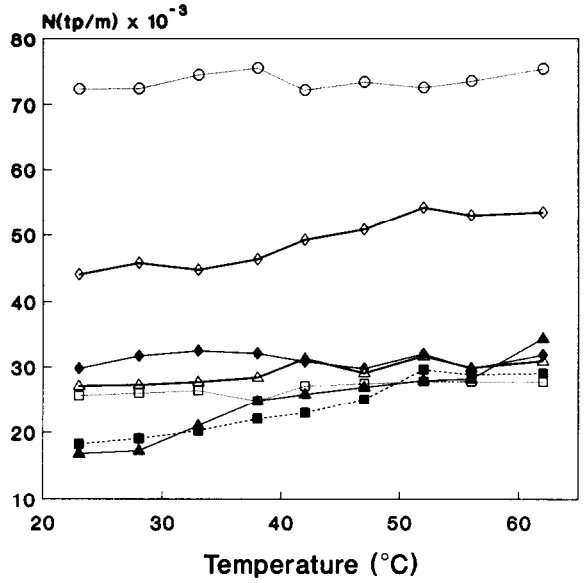

Fig. 3. Effect of temperature on the efficiency. Column, 12 × 0.4 cm I.D. packed with Spherisorb ODS-2 (3 μm); mobile phase, isopropanol–water (9:91) at 0.9 ml/min. N (number of theoretical plates, tp) = 5.54 $(V/W_{\frac{1}{2}})^2$, where V = retention volume of the peak and $W_{\frac{1}{2}}$ = peak width (in volume) at half weight. Symbols as in Fig. 2.

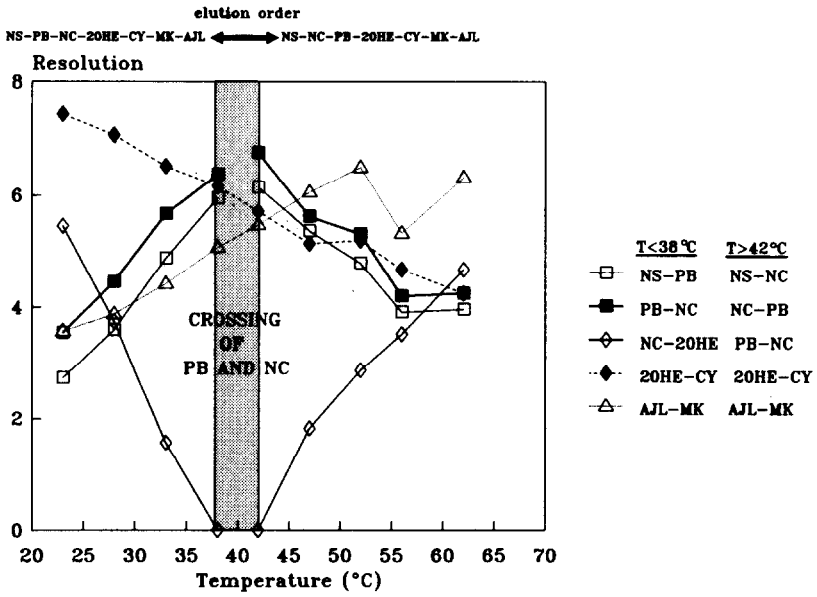

Fig. 4. Variation of the resolution with temperature. The best conditions appear to be in the temperature range 55–60°C. Column, 12 × 0.4 cm I.D. packed with Spherisorb ODS-2 (3 μm); mobile phase, isopropanol–water (9:91) at 0.9 ml/min. $R = (\sqrt{N}/4)[(\alpha-1)/\alpha][k'/(k'-1)]$.

The diminution of the capacity factor has a negative effect on the resolution, but in this instance it is accompanied by a narrowing of the peaks marked enough to produce an increase in the number of theoretical plates. This increment is so important for 29-norsengosterone and polypodine B that the number of plates almost doubled when the temperature was increased from 23 to 60°C. For most of the ecdysteroids, N begins to decrease when the temperature exceeds 50°C (see Fig. 3).

The resolution and selectivity were calculated for pairs of consecutive peaks. As can be seen in Fig. 4, if no peak crossing is involved, the resolution increases with increase in temperature. The discontinuities are due to the crossing of the peaks of 29-norcyasterone and polypodine B, which are not resolved between 35 and 45°C.

The temperature for optimum resolution of all the ecdysteroids was found to be 55°C. At this temperature, under both sets of conditions studied, the value of R was over 3.75 (Fig. 4) and all the peaks were separated near to their bases. With samples that do not contain simultaneously both 29-norcyasterone and polypodine B, optimum resolution was obtained between 40 and 45°C.

The R value for the pair NS–20-HE increased from 7.16 at room temperature to 12.22 at 50°C using the same column (Table II). Moreover, the analysis time was also minimized. The effect of temperature was also studied with the methanol–water mobile phase. An increase in temperature produced a decrease in selectivity and above 30°C 20-hydroxyecdysone was not separated from cyasterone or 29-norcyasterone from polypodine B. In this instance the use of buffers did not result in a better separation (Table II).

After this study, analyses of plants were carried out with isopropanol–water (7:93) at 55°C using he columns described above. Fig. 5 shows the calibration graphs obtained for 20-hydroxyecdysone and ajugalactone. Other ecdysteroids were determined by interpolation on these graphs, taking into account a correction factor corresponding to the different absorptivities. The results found for *P. vulgare* and *Ajuga* species are expressed as percentage of dry weight in Table III.

As no buffer is needed, these conditions might be adapted for preparative or semi-preparative separations. Compounds present in plant extracts together with ecdysteroids show a different behaviour in HPLC with the variation of temperature.

TABLE II

RESOLUTION OBTAINED WITH DIFFERENT TEMPERATURE CONDITIONS

If no buffer is present a clear increase in the resolution is observed from room temperature to 50°C, whereas the use of buffer (formic acid–triethylamine, 0.075 M, pH 3) did not improve it. All analyses were performed using a reversed-phase column, 10 × 0.4 cm I.D.; stationary phase, Spherisorb ODS-2, 5 μm; mobile phase, isopropanol–water (7:93) at 1 ml/min.

Temperature (°C)	*Buffer pH*	*k'*		*α*	*N(BE) (theoretical plates/m)*	*R*
		NS	*20-HE*			
25	–	24.7	36.8	1.49	8000	7.16
25	3	14.5	21.2	1.46	9400	7.29
50	–	15.5	23.6	1.52	22200	12.22
50	3	18.2	12.1	1.47	10300	7.49

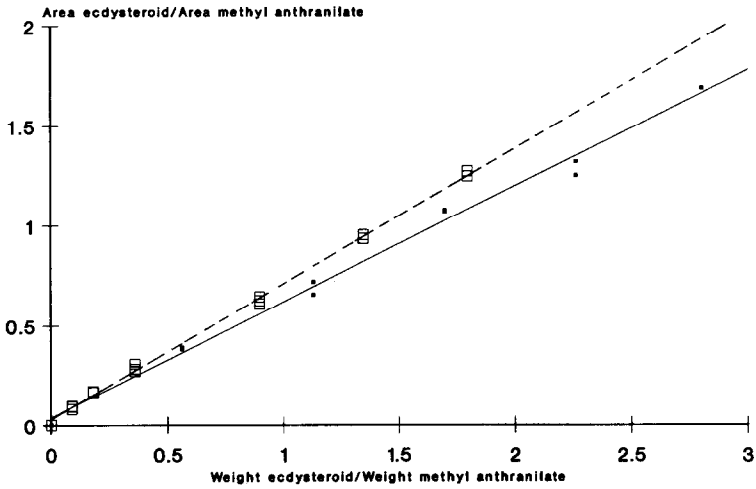

Fig. 5. Linear calibration graphs obtained for 20-hydroxyecdysone (solid line) and ajugalactone (dotted line) with methyl anthranilate as peak-area standard.

Thus, temperature control can be used to optimize the separation conditions around the major peaks to be isolated in each particular problem.

With a semi-preparative column (Tracer Analítica, 15 × 1.0 cm I.D., Spherisorb ODS-2,5 μm) it was possible to obtain 20-hydroxyecdysone or ajugalactone of high purity [300 μg of ecdysteroid (70–85% purity) in 125 μl of methanol in each injection; product recovery 170–200 μg of >99.9% purity by HPLC]. In the same column, up to 8 mg (in 175 μl of methanol) of ecdysteroidal fraction was injected, and the ecdysteroids (>95% pure by HPLC) were recovered in *ca.* 85% of the amount present in the sample.

TABLE III

ECDYSTEROID CONTENTS FOUND IN SOME REAL SAMPLES

Results are ppm dry weight ± S.D. ($n=6$).

Ecdysteroid	*P. vulgare*		*A. reptans*	
	Rhizomes	*Fronds*	*Wild plant*	*Cultivated*
Ecdysone	400 ± 40	300 ± 20		
20-Hydroxyecdysone	4000 ± 200	1200 ± 100	50 ± 20	20 ± 10
Polypodine B	1800 ± 150	100 ± 10		
29-Norsengosterone			210 ± 20	140 ± 20
29-Norcyasterone			450 ± 50	320 ± 30
Cyasterone			110 ± 20	50 ± 10
Ajugalactone			330 ± 20	180 ± 20

CONCLUSIONS

The use of isopropanol–water as eluent in the reversed-phase mode at temperatures above room temperature for the HPLC separation of ecdysteroids proved to be very efficient. The separation of all eight ecdysteroids of this study was accomplished with a short column of only 10 cm. Thermostating of the column allowed a rapid analysis without a decrease in resolution and, in addition, it avoided fluctuations of the retention times of the ecdysteroids. The variation of k' with temperature can be used not only for the optimization of the resolution, but also for identification purposes if the analyses are performed at two different temperatures.

The above conditions allow the quantitative analysis of biological samples that might contain the compounds examined in this work. Further, scale-up to the semi-preparative isolation of pure ecdysteroids has been shown to be readily accomplished. Finally, these conditions can be of great value for the recent liquid chromatograph–mass spectrometer interfaces[15] in order to take maximum advantage of this technique in ecdysteroid analysis.

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