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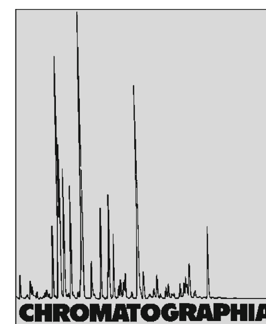
Dynamic On-Column Eluent Modification: A Novel Strategy for Peak Resolution Enhancement. Application to the Preparative Separation of Ecdysteroid Isomers



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Abstract

A novel LC method was applied to enhance the peak resolution of two ecdysteroids: 20-hydroxyecdysone and its 3-epimer. An isocratic solvent system of methanol–water (6:4, *v/v*) was used on a C18 column, and 100 μL of water was injected during the development in such a manner that the eluted solvent peak appeared exactly between the two overlapping peaks. This resulted in the increased retardation of the later-eluted peak, and in a good separation of the two compounds within 4.5 min.

Keywords

Column liquid chromatography
Preparative LC
Ecdysteroid
20-Hydroxyecdysone and 3-epi-20-hydroxyecdysone

Introduction

Phytoecdysteroids are analogues of insect moulting hormones which are widely distributed both in invertebrates and in plants [1]. They exhibit numerous beneficial pharmacological effects: anabolic, adaptogenic, antidepressive, antidiabetic, etc. [2–5]. They are used as inducers in transgenic induced expression systems. Their toxicity in mammals is very low.

Research into these compounds involves three basic areas: (i) the design of ecdysteroid analogues which may possibly serve as new, selective, highly toxic insecticides; (ii) the search for a suitable inducer for the gene expression systems, which act on insect-specific core-receptors that do not occur in mammals; and (iii) the discovery of biologically active ecdysteroids for direct use in human therapy. The isolation of new, effective phytoecdysteroids is an interesting approach to the aims of these research areas.

Ecdysteroids have a steroidal skeleton substituted with a number of hydroxy groups, which furnish them with high polarity. The biosynthetic pathway produces a wide array of ecdysteroid isomers, which differ from each other only in the positions of the hydroxylation, their structures therefore being very similar. Plants contain a complex mixture of structurally closely related ecdysteroids, including major and minor ones [6]. 20-Hydroxyecdysone, the most common ecdysteroid, usually occurs in plants in orders of magnitude higher amounts, than the minor ecdysteroids. Its selective separation is an important requirement for the successful isolation of the minor ecdysteroids, as 20-hydroxyecdysone would otherwise contaminate the entire chromatographic system. Isolation of the minor ecdysteroids demands an extensive prepurification of the extract, followed by multiple steps of chromatographic methods differing in selectivity, including solid phase extraction, extrography, and various types of preparative column chromatography [4, 7, 8].

In the final purification steps, LC is a method of primary importance. The 7-en-6-one chromophore of ecdysteroids makes their UV detection sensitive (log ε ≈ 4 at 240–245 nm).

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A wide range of stationary and mobile phases are used for the LC separation of ecdysteroids [9, 10]. In the NP-LC of these compounds, both adsorbent phases or chemically bonded polar phases, and apolar, chemically bonded stationary phases can be used. Silica, the most widely applied stationary phase, results in outstanding resolution in the separation of ecdysteroids, as confirmed in the literature [11–13]. The most general mobile phases on silica are ternary systems based on dichloromethane, with isopropanol as polar modifier. Many separation problems can be solved by utilizing different ratios of these two organic solvents, but, because of the strong adsorption of ecdysteroids on silica, the peaks display extensive tailing in this case. To obtain symmetrical peaks, water must be added to the solvent system, but this leads to the slow development of equilibrium and gradient elution is not possible. Water slowly adsorbs on the stationary phase, deactivating it, with resulting changes in the retention times, and reducting the reproducibility of the analysis [14]. Isooctane or cyclohexane-based ternary solvent systems with alcoholic modifiers and water are also employed [11, 15].

In RP-LC, chemically modified C8 and C18 phases are widely used [9, 14, 15]. The different mobile phases are greatly affecting the selectivity. Methanol–water and acetonitrile–water are generally the most appropriate solvents, and gradient elution is widely used with both of them [16, 17].

The consecutive use of NP- and RP-LC may allow the achievement of sufficient separation of minor ecdysteroids [14].

Since minor ecdyseroids may be present in the plants in very low amounts, in their isolation the quantitative way has to be targeted. Increasing the purity of fractions by collecting less then the full bands of overlapping compounds is, hence, not preferable. Recyclization is a common technique which may readily solve this problem. An alternative solution is our novel method of dynamic on-column eluent modification. We earlier reported the isolation of a number of ecdysteroids from the plant *Serratula wolffii* where this strategy was successfully used on the NP [18].

In the present paper, we discuss the RP application of the on-column eluent modification in the separation of two overlapping ecdysteroids: 20-hydroxyecdysone (20E; Compound **1**) and 3-epi-20-hydroxyecdysone (3-epi-20E; Compound **2**).

Experimental

Conditions

Chemicals

Methanol of LC grade was obtained from Merck (Darmstadt, Germany). Deionized water was produced with a Leitwertmesser Type 335 apparatus (W. Herrmann u. Co. GmbH, Ludwigsburg, Germany) and was distilled with a rotatory evaporator to obtain water of LC grade.

Equipment

A Jasco PU2080 LC pump and a Jasco UV2075 ultraviolet detector (Jasco Co., Tokyo, Japan) were used, connected to a Hercule 2000 chromatographic interface (JMBS, Grenoble, France).

Detection Parameters

For the detection of ecdysteroids, a wavelength of 245 nm was used. Water was detected at 200 nm when injected separately to determine its elution time. The integrator output scale was 1.0 V/1.0 AU.

Column

An LC column from Agilent Zorbax SB-C18 (Agilent Technologies, Palo Alto, CA, USA), 5 µm, 250 × 4.6 mm was used.

Chromatographic Conditions

Methanol–water (6:4, *v*/*v*) was utilized as the mobile phase eluent at a flow rate of 1 mL min^{-1} at ambient temperature. The optimized mobile phase was methanol–water (48:52, *v*/*v*). A 100 µL loop was used.

Samples

A fraction containing Compounds **1** and **2** was obtained previously by multiple chromatographic separation of the extract of *Serratula wolffii* [18]. By modelling the separation, standard solutions of the isolated compounds were injected in similar amounts to those present in the fraction: 9.35 µg of Compound **1** (5 µL from a 1.87 mg mL^{-1} solution) and/or 9.6 µg of Compound **2** (10 µL from a 0.96 solution), and they were dissolved in methanol–water (6:4, *v*/*v*). By the optimization of the current solvent system, the same concentrations of standard solutions were prepared dissolving them in methanol–water (3:7, *v*/*v*), and the same amounts were injected as described above.

Software

Jasco Borwin v1.50 chromatographic software was used with a measurement frequency of 5 points s^{-1}. Microsoft Excel 2005 was also used. Statistical analysis was carried out by using SPSS for Windows 14.0.

Mathematical Calculations on Chromatographic Data

Each set of absorbance/time coordinates of the chromatograms of the standard solutions was transferred to a ".txt"-file, and data-pairs of these files were translocated to Microsoft Excel. In this way the averages of parallel chromatograms and the superposition of the averages could be calculated and the outcome of the calculations could be visualized as Excel point-diagrams (see Fig. 1b). By plotting the difference quotients of each neighbouring data point of these diagrams, good approximations of the first derivative curves could be obtained. Based on the derivatives, intersections of the tangents of the inflexion points and the time axis could be calculated, giving exact values to the calculation of resolutions.

Implementation of the On-Column Eluent Modification

The elution time of water was determined by a series of injections at 200 nm. The chromatographic software was set to automatic start, and the sample was loaded into the 100 μL loop. When the standards were injected together, they were added one by one, using a 10 μL pipette for Compound **1** and a 20 μL pipette for Compound **2**. After this, the injector was turned into "inject" stage (the recording of the chromatogram was started), and at the same time the stopwatch was set. When the time of the difference between the intersection time of the overlapping ecdysteroid peaks and the time of elution of water was reached, 100 μL of water were injected immediately.

During the isolation of Compounds **1** and **2**, the fractions were collected manually.

Determination of the Overlapping Quantities

The amounts represented by the overlapped areas were calculated via the equations of the calibration lines determined by injecting 18.7, 56.1, 93.5, 130.9, 187.0, 280.5 and 467.5 ng for Compound **1** and 56.1, 93.5, 130.9, 187.0, 280.5, 374.0, 467.5, 748.0 and 935.0 ng for Compound **2**.

Results

Isolation of Compounds 1 and 2 by Using Dynamic On-Column Eluent Modification

The fraction to be separated contained the ecdysteroids Compounds **1** and **2**, which gave overlapping peaks under the given LC conditions. According to our approach, serial measurements were performed to identify the appropriate time of water injection. Its most suitable time was found to be 1.30 min, i.e. the water elution peak is situated between the two peaks at its absorption minimum at 200 nm. Injecting 100 μL of water at this time after the sample injection resulted in baseline separation of the two peaks.

Following this result, Compounds **1** and **2** were successfully isolated. Their structures were elucidated by using various spectroscopic methods, as reported previously [18].

Modelling of the Separation

As a further outcome of our serial measurements of water injections it could also be concluded, that the injected solvent not only increases the separation of the overlapping peaks of the two compounds, but it may also duplicate a single peak. This suggested, that although the separation achieved by the adequate timed injection of water gave a baseline separation, some quantities of both compounds may have remained overlapped.

To examine this phenomenon, a virtual separation was performed with chromatograms obtained with standard solutions of the two isolated ecdysteroids in separate experiments. The same amounts of both compounds were developed in each experiment, as they were present in the original fraction from which they were isolated. Five parallel runs were carried out with Compounds **1** and **2** separately, then five separate runs with each compound injecting 100 μL of water at 1.30 min. Two runs of the co-injected standards with the injection of 100 μL of water at 1.30 min were also performed. One of these two experiments was performed before the four series of runs with the standards, and the other one afterwards.

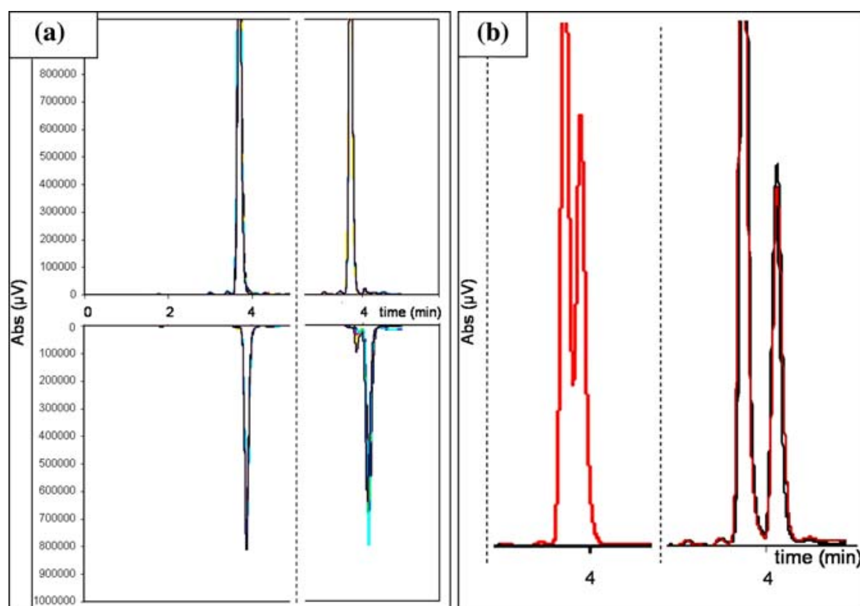

Fig. 1. Modelling the separation. **a** The four series of the five–five separately developed chromatograms of Compounds **1** and **2** with and without our novel approach. **b** Virtual chromatograms obtained by mathematical calculation of the former chromatograms (red/grey line, online/print). Real chromatograms of the co-injected compounds separated by injecting 100 μL of water at 1.30 min are also represented (black lines), highly corresponding to the virtual ones. The mobile phase was methanol:water (6:4, *v*/*v*) at a flow rate of 1 mL min^{-1}

Figure 1a shows each of the four series of separately developed chromatograms (Compounds **1** and **2** without on-column eluent modification, and Compounds **1** and **2** with the use of it). Each series was averaged and the corresponding averages were superpositioned as described above, to construct a virtual separation (Fig. 1b). The superposition calculated by mathematical summation of the data (red/grey line, online/print) corresponds to the chromatograms of the two co-injected compounds separated by using our novel method (black lines).

Figure 1 reveals that to the achievement of the maximum purity and recovery for both compounds, the ideal

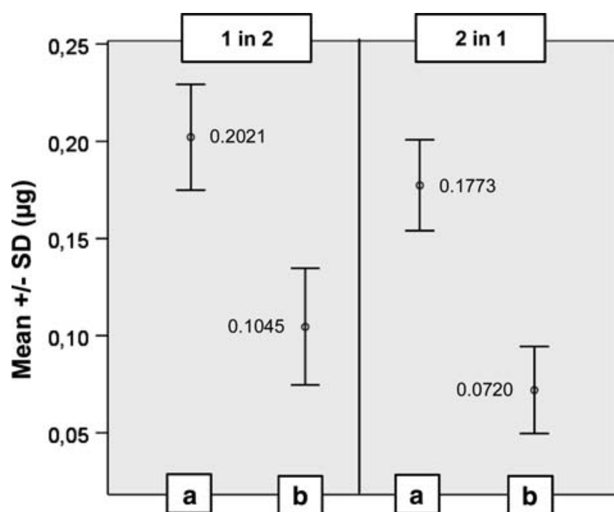

Fig. 2. Comparison of the separation efficacy obtained by the conventional method (**a**) and by using on-column eluent modification (**b**). The left side error bar shows the amount of Compound **1** remaining overlapped with Compound **2** in µg, and the right side shows the reverse case

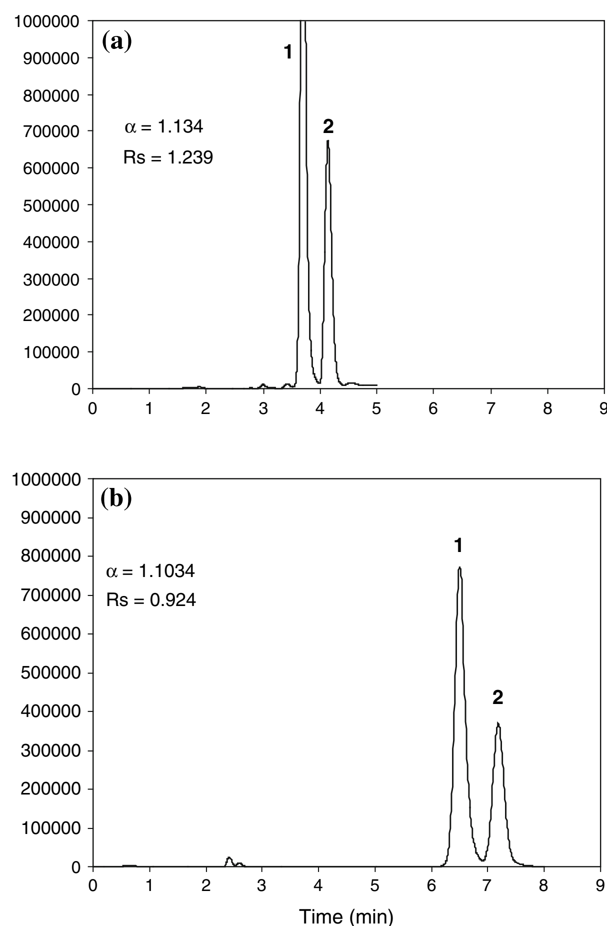

Fig. 3. Comparison of the separation obtained by using our method and an optimized solvent system. **a** The superposition of the averaged chromatogram series of standards with the use methanol:water (6:4, *v*/*v*) as mobile phase and using on-column eluent modification. **b** The chromatogram of the co-injected standards of Compounds **1** and **2** by using the optimized solvent system of methanol:water (48:52, *v*/*v*). Selectivity factors (α) and resolutions (R_s) are also represented

time for a change in fraction collecting is somewhat earlier than the minimum in the superpositioned curve.

The data were evaluated from a chromatographic aspect. Each of the four series of chromatograms obtained by developing the standards were aligned according to the retention times, and the corresponding ones were paired. Data of chromatogram pairs obtained by using the conventional way are as follows ($t_R^{20E}/t_R^{3-epi-20E}$ {α, R_s}, respectively): 3.660/3.890 {1.063, 0.629}; 3.660/3.893 {1.064, 0.633}; 3.663/3.893 {1.063, 0.628}; 3.670/3.897 {1.062, 0.623}; 3.677/3.900 {1.061, 0.611}. The corresponding data of our concept are: 3.653/4.143 {1.134, 1.215}; 3.660/4.147 {1.133, 1.200}; 3.663/4.147 {1.132, 1.244}; 3.663/4.150 {1.133, 1.319}; 3.663/4.167 {1.138, 1.217}. These results give the average values of 3.666/3.895 {1.062, 0.625} for the conventional separation, and 3.660/4.151 {1.134, 1.239} for that of obtained by using dynamic on-column eluent modification, indicating significant improvement of the separation.

The overlapping areas for each of the chromatogram pairs were calculated as follows:

- The time to the intersection of the two peaks was determined.
- Integration was carried out from the intersection to the end for the first-eluted peak, and from the beginning to the intersection for the later-eluting one.

The amounts represented by these overlapped areas were taken into consideration as the loss in one peak and as the contamination of the other.

Determination of the Overlapping Quantities and Statistical Analysis

The overlapping quantities were almost two orders of magnitude less than the amounts injected, and therefore calibration lines were determined for both Compound **1** (n = 7) and Compound **2** (n = 9) in the appropriate concentration range in order to calculate them most accurately.

Table 1. Comparison of the separation of Compounds **1** and **2** for the selected chromatogram-pairs of standards developed in the conventional way (**a**) and by using our concept (**b**)

	Compound **1**				Compound **2**			
	Yield (%)		Contamination (%)		Yield (%)		Contamination (%)	
	a	b	a	b	a	b	a	b
1	98.04	98.89	1.96	1.11	95.26	97.39	4.74	2.61
2	98.27	99.13	1.73	0.87	95.73	97.05	4.29	2.95
3	97.90	99.50	2.10	0.50	95.75	97.63	4.25	2.37
4	98.43	99.41	1.57	0.59	96.55	98.67	3.44	1.33
5	97.85	99.22	2.15	0.78	95.09	98.11	4.91	1.89
Mean (±SD)	98.10 (±0.25)	99.23 (±0.24)	1.90 (±0.25)	0.77 (±0.24)	95.68 (±0.58)	97.77 (±0.63)	4.32 (±0.58)	2.23 (±0.63)

Values are given in m/m %

The equations of the calibration lines with the regression coefficients and standard errors were

$$Y = 8 \times 10^{-7}X - 0.0038 \quad (R^2 = 0.9993;\ \mathrm{SE} = 0.0044)$$

for Compound **1**, and

$$Y = 8 \times 10^{-7}X - 0.0201 \quad (R^2 = 0.9961;\ \mathrm{SE} = 0.0103)$$

for Compound **2**.

The differences between the effectivities of the conventional chromatographic technique and that of our method as concerns the overlapping quantities of the two compounds were examined with one-sample *T*-tests at a significance level of 95%. Figure 2 illustrates the results of the analysis. For both compounds, a significant decrease of the contamination could be achieved with the use of on-column eluent modification.

The accessible yields and purities (if the two ecdysteroids had been co-injected and separated at the intersection time) of both compounds were calculated by using the amounts injected and the overlapping quantities obtained from the calibration, according to the formula:

$$\text{Contamination }\%_{\text{comp. 1}} = \left[1 - \left(m_{\text{inj. comp. 1}} - m_{\text{lost comp. 1}}\right) \times \left(m_{\text{inj. comp. 1}} - m_{\text{lost comp. 1}} + m_{\text{lost comp. 2}}\right)^{-1}\right] \times 100$$

The results are shown in Table 1.

Optimization of the Current Isocratic System

The separation of the partially resolved mixture of the two compounds can certainly also be increased by lowering the organic modifier content of the existing mobile phase. Therefore, to see the benefit of our approach more clearly, the current isocratic system was optimized. The same amounts were injected for both compounds as described previously, in a 30% methanol solution. The solvent system of methanol:water (48:52, *v*/*v*) was found to give a baseline-separation, as is depicted in Fig. 3.

Discussion

The results demonstrated, that the overlapping areas were significantly smaller when dynamic on-column eluent change was used, comparing to that of obtained by using the same isocratic system in the conventional way.

The manual collection of fractions is also easier if our method is applied since the slope of the chromatogram between the two peaks is much lower in this case. By using the optimized solvent system of methanol:water (48:52, *v*/*v*) a complete resolution could be achieved, but our approach offers an alternative to this with a significantly shorter separation time. This benefit may have particular importance in the isolation from complex biological samples (e.g. only partially prepurified plant extracts) containing further constituents with longer retention time.

We suggest the following mechanism of the increased separation: Water is the solvent component with the lowest elution force on the reverse phase. It suffers minimal retention, and passes through the column quickly. Wherever it passes, it changes the equilibrium between the flowing solvent and the thin static solvent layer around the microenvironment of the particles. This results in a higher retention of the affected sample components. Accordingly, if the injected solvent is eluted between two overlapping peaks, the main quantity of the later-eluting component of the sample suffers stronger retention. Moreover, neither the change in the equilibrium nor the re-equilibration are instant, so two gradients are generated near the surface (one with a weakening and the other with a normalizing solvent force). It is suggested that these gradients will increase the separation of the two components in the overlapping zone.

On the basis of the suggested mechanism our approach may be appropriate not only in the isolation of ecdysteroids, but also for other compounds. However, the questions according to the role of different volumes of the injected solvent in the efficiency of the method and in the scale-up remain a topic of further research.

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