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Computer-aided optimization of high-performance liquid chromatographic analysis of flavonoids from some species of the genus *Althaea*

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ABSTRACT

DryLab G software was used for the optimization of gradient elution programs in reversed-phase high-performance liquid chromatography applied to the chromatographic analysis of flavonoids present in marsh-mallow (*Althaea officinalis*). The optimization experiments were carried out for a mixture of eight standard solutes (isolated previously from the plant) and then applied to an extract from the flowers of marsh-mallow. Computer simulation experiments allowed convenient analytical conditions to be chosen. The use of two modifiers, methanol and acetonitrile, made the identification of separated components more certain. Good agreement between simulated and experimental chromatograms was obtained.

INTRODUCTION

Several species of the genus *Althaea* grow in Europe [1]: in Poland the marsh-mallow (*Althaea officinalis* L.) is grown as a medicinal plant. The leaves, flowers and roots of marsh-mallow are used to prepare anti-inflammatory and mucilaginous drugs. In the USSR other *Althaea* species are also used in the production of drugs [2].

In previous papers [3,4] the content of phenolic acids, coumarins and flavonoid glycosides in several species of *Althaea* (*A. officinalis* L., *A. armeniaca* Ten., *A. cannabina* L., *A. narbonensis* Pourr. and *A. broussenetiifolia* Iljin) were investigated by means of reversed-phase high-performance liquid chromatography (RP-HPLC) and paper chromatography; other examples of separation and determination of flavonoids can also be cited [5–7]. The analysis of flavonoids is more difficult owing to the larger number of components, therefore the chromatographic systems were optimized in preliminary experiments using the DryLab G software (LC Resources, Lafayette, CA, USA; I. Molnar, Institute of Applied Chromatography, Berlin, Germany) [8,9]. The use of gradient elution is also advantageous because it rapidly removes the less polar ballast substances present in the biological sample.

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EXPERIMENTAL

DryLab G software was applied using an IBM class computer. HPLC experiments were carried out using a Hewlett-Packard (Palo Alto, CA, USA) Model 1050 liquid chromatograph with a 20-μl sample injector (Rheodyne, Cotati, CA, USA) and a spectrophotometric detector. The chromatograms were recorded at 270 nm with a 3396A reporting integrator (Hewlett-Packard). The stainless-steel column, 250 × 4.6 mm I.D., was packed with 7-μm LiChrosorb RP-18 (E. Merck, Darmstadt, Germany) with a home-made apparatus (Orlita pump). Samples of 10 μl were injected. Eluents contained 1% acetic acid in the whole composition ranges and were composed of bidistilled water and methanol or acetonitrile, chromatographic grade (E. Merck). The column dead volume, 2.3 cm^3, was determined using a pure water sample for eluent composed of methanol and water (60:40). The column efficiency was 7300 theoretical plates determined for benzene and toluene at methanol–water (60:40), flow-rate 1 cm^3/min, temperature, 20°C.

The gradient program was optimized using an artifical mixture of eight standard solutes isolated previously from the plant.

The plant material (flowers, 1.0 g), after extraction with chloroform, was extracted with boiling methanol (5 × 50 ml). Combined methanol extracts were evaporated to 100 ml *in vacuo*.

RESULTS AND DISCUSSION

According to the routine procedure [8,9] two gradient runs were carried out at different gradient steepness (gradient times for 5–100% acetonitrile–water: 20 and 60 min, Fig. 1a,b).

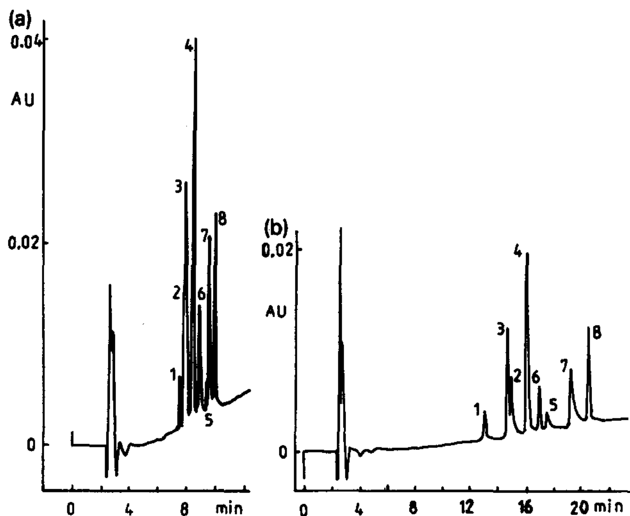

Fig. 1. Separation of the test flavonoid compounds by gradient elution with 5–100% acetonitrile–water. Peaks: 1 = dihydrokaempferol 4'-O-glucoside; 2 = hypolaetin 8-O-gentiobioside; 3 = quercetin 3-O-glucoside; 4 = kaempferol 3-O-glucoside; 5 = hypolaetin 8-O-glucoside; 6 = naryngenin 4'-O-glucoside; 7 = hypolaetin 4'-methyl ether 8-O-glucoside; 8 = tiliroside. Gradient time (a) 20 min, (b) 60 min.

TABLE I

RETENTION DATA

System variables: dwell volume, 0.45 ml; column length, 25.00 × 0.46 cm I.D.; flow-rate, 1.00 cm^3/min; starting percentage —B, 5.00%; final percentage —B, 100.00%; gradient time, first run, 20.00 min; gradient time, second run, 60.00 min. t_R = Retention time.

Band	Solute	Run 1		Run 2: t_R (min)
		t_R (min)	Area (arbitrary units)	
1	Dihydrokaempferol 4′-O-glucoside	7.64	111000.00	13.10
2	Hypolaetin 8-O-gentiobioside	7.89	334000.00	14.91
3	Quercetin 3-O-glucoside	7.99	808000.00	14.63
4	Kaempferol 3-O-glucoside	8.50	999999.00	16.03
5	Hypolaetin 8-O-glucoside	8.82	70000.000	17.47
6	Naryngenin 4′-O-glucoside	8.97	500000.00	16.92
7	Hypolaetin 4′-methyl ether 8-O-glucoside	9.65	750000.00	19.22
8	Tiliroside	10.09	690000.00	20.49

The retention data for the eight solutes, summarized in Table I, show a changed sequence of two pairs of solutes, Nos. 2–3 and 5–6, in run 2. The resolution plot calculated by the DryLab G software from the data is given in Fig. 2. It can be seen that good resolution of all solute pairs is obtained at rather long gradient times (*ca.* 80 min). Considering the long retention times of the first components (Table I), several gradient runs were simulated by means of option 6.4 (change of gradient range),

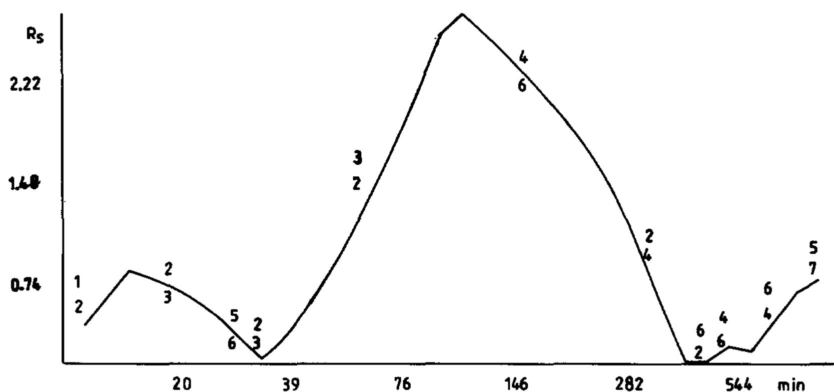

Fig. 2. Relative-resolution map for flavonoid compounds, 5–100% acetonitrile–water gradients and compounds as in Fig. 1.

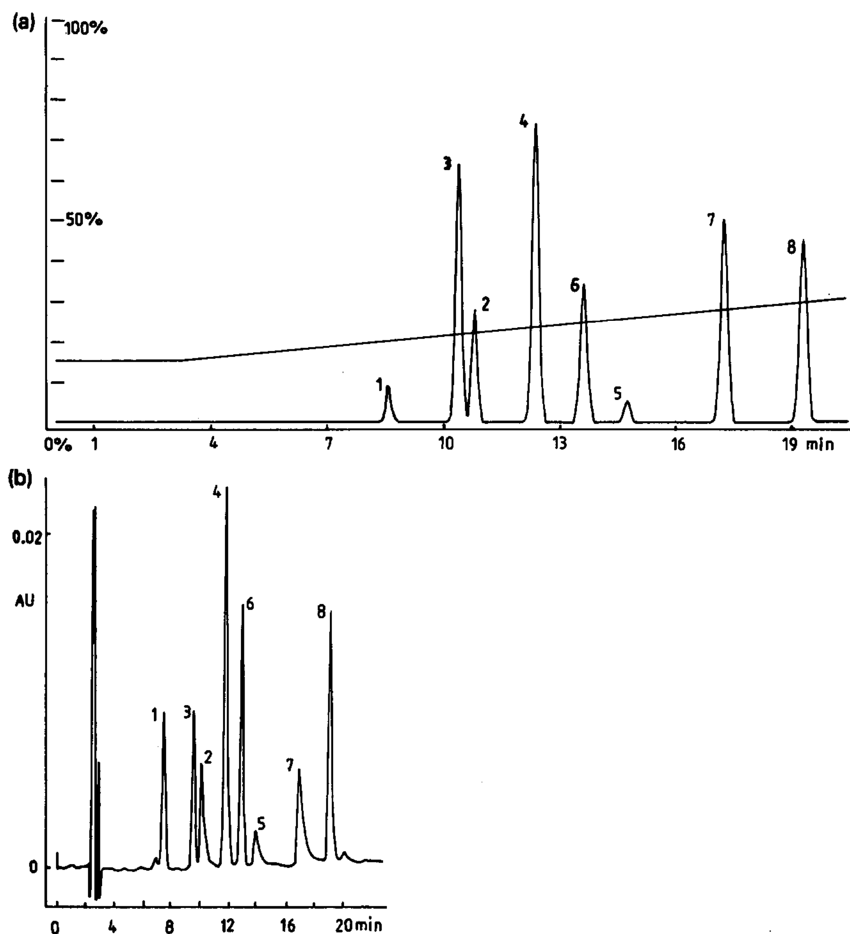

Fig. 3. (a) Computer-simulated chromatogram of the test mixture of flavonoid compounds (as in Fig. 1a), gradient elution with 16–35% acetonitrile–water, gradient time 22 min. (b) Experimental chromatogram of the mixture, sample as in Figs. 1a and 3a.

starting from a higher initial concentration of acetonitrile; good results were obtained using a 16 to 35% acetonitrile (1% acetic acid) linear gradient in 22 min. The simulated chromatogram of the eight standards, after option 7 (column optimization, assuming molecular weight, $M = 700$), is shown in Fig. 3a and the experimental chromatogram in Fig. 3b. Satisfactory separation and good agreement of simulated and experimental chromatograms can be seen.

In a similar way another solvent system, containing methanol instead of acetonitrile, was optimized. Satisfactory separation of all eight standards was obtained for a linear 29 to 65% methanol gradient (1% acetic acid) and 21 min gradient time (Fig. 4), also in good agreement with the simulated chromatogram. The separation is, however, somewhat worse than for the acetonitrile system (Fig. 3); the sequence of the solutes is quite different for the two systems, which may be advantageous for further improvement of separation by the use of ternary gradients.

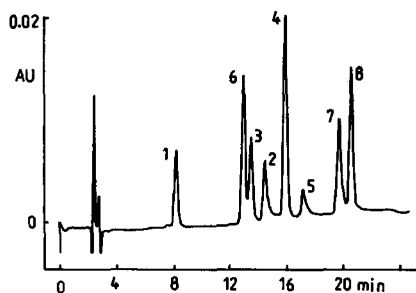

Fig. 4. Experimental chromatogram of the test mixture as in Fig. 1a, gradient elution with 29–65% methanol in water, gradient time 21 min. and compounds as in Fig. 1a.

The gradient programs were then used for the separation of actual flavonoid extracts from the flowers of *Althaea officinalis* L. (Fig. 5a acetronitrile and Fig. 5b methanol systems) and *A. cannabina* . (Fig. 6, methanol system). The flavonoid com-

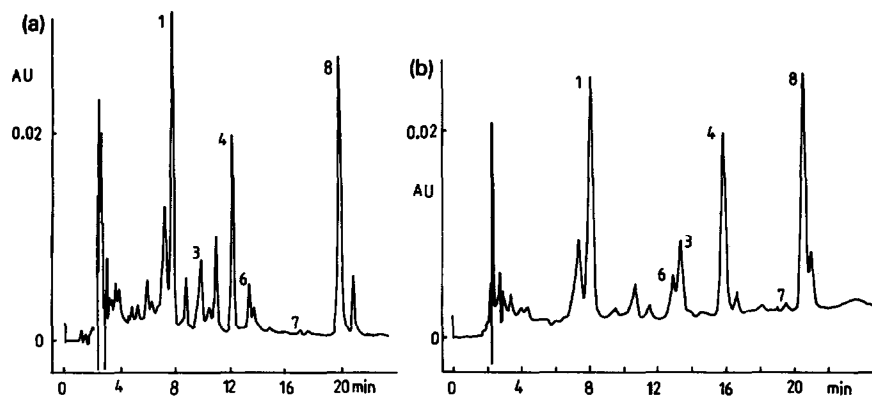

Fig. 5. Experimental chromatogram of the actual flavonoid extracts from the flowers of *Althaea officinalis* L. (a) Conditions as in Fig. 3, (b) as in Fig. 4.

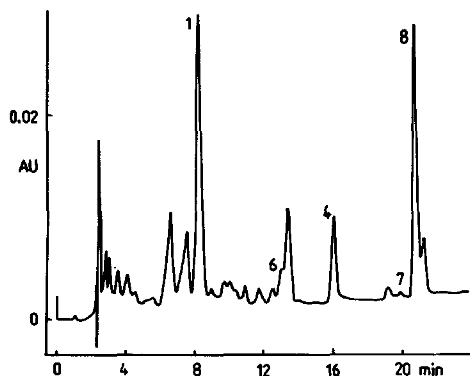

Fig. 6. Experimental chromatogram of the actual flavonoid extract from the flowers of *Althaea cannabina* L. Conditions as in Fig. 4.

position is different from the standard mixtures (Figs. 3 and 4): some new peaks are present and some of the standards occur in trace amounts. However, some compounds could be positively identified in the chromatograms of the extracts, and the identification could be more certain because of the use of two modifiers, methanol and acetonitrile, having different selectivity characteristics.

The analytical applications of the optimized systems, calibration experiments and comparison of flavonoid content in various species of the *Althaea* genus will be described in a later paper.

CONCLUSIONS

The use of DryLab G software for the optimization of gradient programs permitted the choice of convenient experimental conditions for the rapid separation and quantitation of components of complex plant extracts, basing on a few experiments. For the more reliable identification of separated components it is advantageous to use more than one eluent system (modifier).

Good agreement of simulated and experimental chromatograms was observed.

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