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ANALYSIS OF FLAVONOIDS IN *Silybum marianum* FRUIT BY HPLC

R. A. Minakhmetov,[1] L. A. Onuchak,[1] V. A. Kurkin,[2] E. V. Avdeeva,[2] and A. V. Volotsueva[2]

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*Flavonoids of spotted milkweed (*Silybum marianum *L. Gaerth.) were optimally separated using reverse-phase HPLC with isocratic elution by $CH_3CN—H_2O$ over Separon SGX C_{18}. The flavolignans silybin, silydianin, silychristin, and the flavonoid taxifolin were identified in the chromatograms of extracts from this plant.*

Key words: *Silybum marianum*, flavonoids, flavolignans, silybin, silydianin, silychristin, taxifolin, micro-column HPLC.

Fruit of spotted milkweed (*Silybum marianum* L. Gaerth., Asteraceae, aster or composite) is used widely to produce such valuable hepatoprotector preparations as carsil, legalon, siliborum, sibectanum, silimarum, etc. [1-3]. Their pharmacological action is due to hepatoprotective properties of a new group of biologically active compounds, flavolignans [2, 4, 5]. It should be emphasized that medicinal compounds based on spotted milkweed are necessary not only for liver treatment but also for prophalyxis of various illnesses resulting from the effects of unfavorable environmental factors on the organism [6].

Research on new medicines based on spotted milkweed fruit is urgently needed. The Samara district has enormous capabilities for developing new domestic preparations based on the flavolignans of spotted milkweed. The largest industrial source of this plant is located there.

Collaboration of scientists of Samara State Medical University, All-Russian Scientific-Research Institute of Medicinal and Aromatic Plants, and staff of the ZAO Samara Pharmaceutical Plant developed new hepatoprotectors based on fruit of *Silybum marianum* L., liquid extract of milkweed and silibochol [1]. This necessitated the improvement of existing analytical methods for fruit of *Silybum marianum* L.

According to the standards for spotted milkweed fruit (TY 64-4-97-93), quantitative determination of flavolignans is carried out by differential UV spectrophotometry. We propose as an alternative direct spectrophotometry using a State Sample of silybin [7]. It should be noted that the total flavolignans is determined by these methods. A more reliable and rapid analytical method for medicinal plant material is high-pressure liquid chromatography (HPLC), which combines the separation of substances and the ability to determine them qualitatively and quantitatively.

An attempt was made previously to use HPLC for quantitative estimation of the silybin and total flavolignan content [7]. However, optimal resolution of the components of spotted milkweed fruit was not obtained. Successful separation, identification, and quantitative determination of the components was achieved using a reverse-phase sorbent and $CH_3OH—H_2O$ eluent with added acetic acid [8, 9]. However, the high viscosity of the binary eluent $CH_3OH—H_2O$ makes the experiment difficult on an HPLC microcolumn. In our opinion, a mobile phase based on $CH_3CN—H_2O$ is preferable. Reverse-phase HPLC using this eluent was successfully applied to the analysis of taragon (*Artemisia dracunculus* L.) extracts [10].

Our goal was to develop a method for qualitative analysis of flavonoids in spotted milkweed fruit using an HPLC microcolumn and $CH_3CN—H_2O$ mobile phase.

We studied industrial samples of spotted milkweed collected in Samara district (Sergievskii collective farm).

The standards were pure flavolignans silybin (**1**), silydianin (**2**), silychristin (**3**), and 2,3-dehydrosilybin. These were isolated from medical raw material by preparative column chromatography and identified by UV and NMR spectroscopies and mass spectrometry [7, 11]. A sample of the flavonoid taxifolin (dihydroquercetin) (**4**) was graciously supplied by Dr. Prof. N. A. Tyukavkina.

1) Samara State University, 443011, Russia, Samara, fax (8462) 34 54 17, e-mail: onuchak@ssu.samara.ru; 2) Samara State Medical University, 443099, Russia, Samara, fax (8462) 33 29 76, e-mail: vakur@samaramail.ru. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 272-274, July-August, 2001. Original article submitted August 27, 2001.

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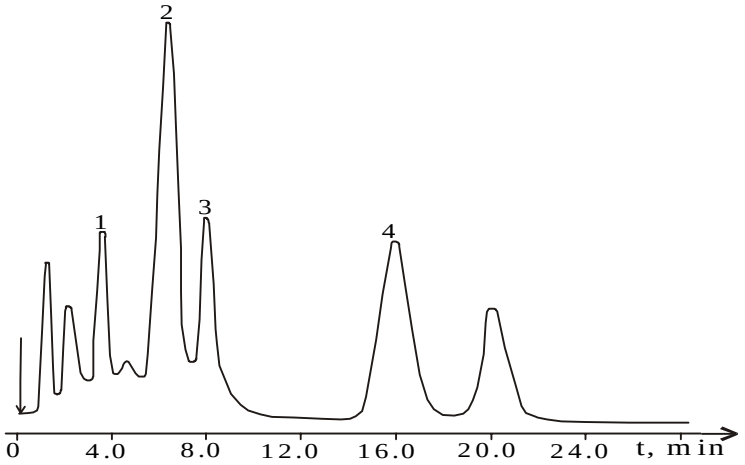
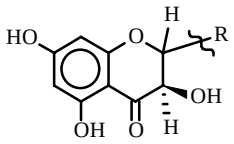


Fig. 1. Chromatogram of extract from *Silybum marianum* fruit. Column: steel, 120×2 mm; stationary phase: Separon SGX C_{18}; eluent flow rate F_c = 200 μL/min; detector UV spectrophotometer, λ = 290 nm; eluent CH_3CN—H_2O (27:73 vol. %, pH = 3.0). Taxifolin (1), silychristin (2), silydianin (3), silybin (4).



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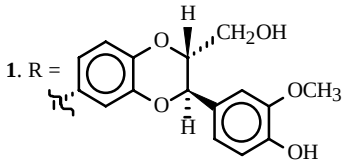

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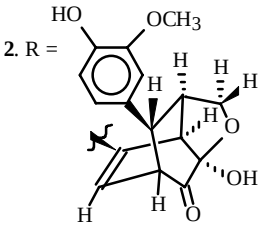

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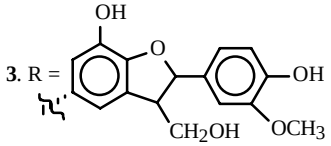

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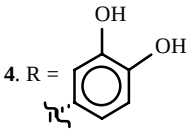

4. R =

Extracts of spotted milkweed fruit were analyzed by reverse-phase HPLC. A microcolumn was packed with Separon SGX C_{18} adsorbent. The mobile phase consisted of CH_3CN, H_2O, and CH_3CO_2H. The components were optimally resolved with CH_3CN concentrations in the aqueous eluent varying from 25 to 40 vol. % and pH values from 2.5 to 4.0.

It was found that decreasing the content of the organic modifier (CH_3CN) to 27 vol. % increases the retention of the analytes. This improves the resolution of the flavonoid components because the intermolecular interactions of the analytes with the organic modifier weaken as the CH_3CN concentration decreases. This causes them to interact selectively with the sorbent. Compounds **1**-**3** are retained more strongly than **4** owing to the stronger attraction to the nonpolar adsorbent.

Concentrations of CH_3CN in the range from 27 to 30 vol. % are optimal for separating the studied compounds. Increasing the content of the organic modifier above 30 vol. % causes a sharp drop in the selectivity. Decreasing the CH_3CN content below 27 vol. % greatly broadens the peaks but retains the selectivity.

Another factor that affects the selectivity of the separation is the pH of the mobile phase. Decreasing the pH from 4.0 to 3.0 improves the separation of the components. However, decreasing the pH further from 3.0 to 2.5 does not noticeably increase the selectivity.

The optimum separation conditions were found: CH_3CN—H_2O (27:73 vol. %, pH = 3.0) (Fig. 1).

Compounds **1**-**4** were identified in the chromatogram of spotted milkweed fruit extract by comparing t_R and k of the peaks (Table 1). Standards were also added directly to the plant extract. This increased the heights of the corresponding peaks. It should be emphasized that the silybin derivative - dehyhdrosilybin that was discussed in the literature [8] was not eluted from the column under our conditions.

TABLE 1. Retention Times and Factors of Flavonoids from *Silybum marianum* Fruit on Separon SGX C_{18} (CH_3CN—H_2O Eluent, 27:73 vol. %, pH = 3.0)

CH_3CN conc., vol. %	Taxifolin		Silychristin		Silydianin		Silybin	
	t_R, min	k	t_R, min	k	t_R, min	k	t_R, min	k
27	3.35	1.62	5.53	3.32	7.25	4.65	16.83	12.15
28	2.34	0.83	4.72	2.69	5.57	3.35	12.78	8.98
30	2.30	0.80	4.35	2.40	5.17	3.04	9.70	6.58

We note that the degree of separation of the poorly separated pair of compounds silydianin and silychristin is greater (R_S = 0.94) with microcolumn liquid chromatography using an eluent of the optimum composition (CH_3CN—H_2O, 27:73 vol. %, pH = 3.0) than that (R_S = 0.70) calculated from the chromatogram published previously [8]. Furthermore, the duration of the analysis is significantly shorter for microcolumn HPLC.

Thus, the optimal resolution of flavonoid components of spotted milkweed components is achieved and silybin, silydianin, silychristin, and taxifolin are identified.

EXPERIMENTAL

Preparation of Spotted Milkweed Fruit Extract. A portion of air-dried spotted milkweed was extracted with ethanol (95%) according to TU 64-4-97-93 (triple extraction at a 1:50 ratio with boiling for 30 min). The volume of the combined ethanol extracts was adjusted with ethanol (95%) to 200 mL. The resulting extract was diluted 1:20 with CH_3CN—H_2O eluent (27:73 vol. %, pH = 3.0), passed through a NF-13 membrane filter, and introduced into the chromatograph.

Chromatographic Analysis of Spotted Milkweed Fruit Extracts. We performed reverse-phase HPLC on a microcolumn in a Milikhrom-1 chromatograph with a modified mobile-phase delivery system using a Gilson (France) pump and a six-port valve-injector, a 120×2 mm steel column packed with Separon SGX C_{18} adsorbent (Elsiko, Moscow) of particle size 7 μm. Eluents were binary solutions of CH_3CN (from 25 to 40 vol. %) in H_2O with conc. CH_3CO_2H added to give the necessary pH. The pH range of the mobile phases was 2.5÷4.0. The experimental conditions were: mobile-phase flow rate F_C = 200 μL/min, detector wavelength λ = 290 nm, 10 μL sample size. Eluent was prepared by mixing pure CH_3CN and H_2O, adjusting the appropriate pH value using conc. CH_3CO_2H, and treating the prepared mixture in an ultrasonicator to remove dissolved gas. Impurities were removed by filtering through a NF-13 membrane filter.

Retention times t_R and factors k were calculated from the chromatograms using the formula:

$$k = (t_R - t_M)/t_M, \quad (1)$$

where t_R is the retention time of the analyte and t_M is the residence time in the column of a nonadsorbed substance ($NaNO_2$). The peak resolution R_S (degree of separation) of silydianin and silychristin was:

$$R_S = (t_{R,2} - t_{R,1})/(\tau_{h,1} + \tau_{h,2}), \quad (2)$$

where $t_{R,2}$ and $t_{R,1}$ are the retention times of silydianin and silychristin, respectively; $\tau_{h,1}$ and $\tau_{h,2}$ are the half-widths of the silydianin and silychristin peaks expressed in time units.

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