We have studied the extraction of *Trifolium pratense* L. herbs using aqueous ethanol solutions of various concentration and biphasic systems containing volatile solvents. Dependences of the yield of the active substances on the extractant composition and process factors have been studied. It is established that the maximum total yield of biologically active substances from *Trifolium pratense* herbs is obtained with the use of biphasic solvent systems.

Flavonoids are the principal active substances of *Trifolium pratense*. Various researchers have shown that the total flavonoid preparation and total isoflavones of red clover could lower the cholesterol content in blood by more than 50% for experimental hypercholesterolemia and atherosclerosis in animal tests [1], exhibit anti-arrhythmic activity for arrhythmia [2], and stimulate peristalsis of the intestinal tract [3].

Thus, total flavonoids of *T. pratense* are promising substances for pharmaceutical use. Herein we study the possibility of increasing the yield of flavonoids from raw material by selecting the optimum extractant for their extraction.

Aqueous ethanol solutions or hot water is used most often to produce extracts of *T. pratense* herb. Alcohol of various concentrations and water extract most flavonoids [4].

We evaluated the resulting extracts for content of total flavonols. It was determined preliminarily that the rate and degree of extraction of isoflavonoids and flavonols by aqueous alcohol extractants are approximately the same and that their mass ratio in the extract corresponds with the mass ratio in the raw material.

Thus, it seemed possible to evaluate the extraction capacity of extractants for one group of substances (marker) relative to related groups. Flavonols for which the content in the extract could be easily evaluated using differential spectrophotometry calculated for rutin using one of the standard methods were selected as such a marker [5].

Paper and thin-layer chromatography were used to study and identify biologically active compounds (BAC) in the resulting extracts.

Using aqueous sodium carbonate (5%), which reacts with flavonoids, as the mobile phase turned out to be a successful new method of paper chromatography. The sensitivity of the analysis, the intensity of the color, and the stability of the spots increased. The spectrum of determined compounds expanded.

Bands of compounds with different colors were obtained on the chromatograms. The isoflavonoid band, which was pale green, was the brightest and largest. Two other bands were also clearly visible. These were the flavonol band, which was yellow, and the chalcone band, which was red (Fig. 1). The color of the chromatogram did not fade with time. Also, the red color could not be observed upon development with sodium carbonate of chromatograms obtained in other systems used to separate flavonoids. It should be noted that we could not find literature references to the presence of chalcones in *Trifolium* spp.

This leads to the conclusion that the separation system proposed by us, aqueous sodium carbonate (5%), is selective for various flavonoid classes and can be used successfully for qualitative evaluation of the BAC composition of *T. pratense* herb.
We also found that isoflavonoids are separated most completely on TLC by benzene:methanol (4:1).

We used the following solvents for extraction of BAC from *Trifolium pratense* herb: ethanol of various concentrations (25%, 40, 50, 70, 96); biphasic systems of isobutanol:ethanol:water, CH\textsubscript{2}Cl\textsubscript{2}:ethanol:water, dichloroethane:ethanol:water, and CCl\textsubscript{4}:ethanol:water in various ratios.

We performed liquid—liquid extraction for purification of the alcohol extracts from a large number of accompanying ballast substances (chlorophyll, resins, etc.).

For this, the alcohol extract was evaporated in vacuo to remove completely the alcohol. Flavonoids were extracted 3 – 4 times with ethylacetate to extract them completely from the aqueous phase. The completeness of the extraction was determined using color reactions for flavonoids. Because ethylacetate is a selective extractant for flavonoids, back-extraction produced total flavonoids with an insignificant amount of accompanying substances.

A sample of the ethylacetate back-extract was placed on a chromatogram. The number of spots was determined (Fig. 2).

Qualitative and quantitative analysis using TLC and differential spectroscopy showed that use of ethanol (70%) as extractant gave the most complete extraction of total flavonoids.

Extracts were obtained by percolation with preliminary soaking. It is known that the particle size of medicinal plant raw material has a substantial effect on the completeness of BAC extraction.

**TABLE 1. Flavonol Content in *Trifolium pratense* Extracts as a Function of Particle Size**

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<th>Particle size, mm</th>
<th>Flavonol content, %</th>
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<tr>
<td>&gt; 3 mm</td>
<td>0.096 ± 0.003</td>
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Fig. 1. Paper chromatograms of *Trifolium pratense* extract using sodium carbonate (5% aqueous solution).

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We found that the maximum yield of flavonols and, therefore, other flavonoid classes from *Trifolium pratense* was reached using raw material with particle size from 0.5 to 1.0 mm (Table 1).

It was also found that the maximum yield of flavonoids was reached for the percolation method with preliminary soaking of raw material for 6 – 12 h.

Thus, the optimum parameters for extraction by aqueous alcohol extractants were ethanol (70%), raw material of particle size 0.5 – 1 mm, and preliminary soaking for 6 – 12 h. The flavonoid yield was 40 – 43%, which is a comparatively low value for the percolation method.

The flavonoid yield was 55% for the maceration method with stirring (Fig. 3).

Extraction with biphasic extractants was carried out with stirring (10 g of loaded raw material). *Trifolium pratense* herb was treated beforehand with the prepared extractant system at a 1:20 ratio (Fig. 3). The extraction was carried out with stirring for 60 min. The extract was filtered. The phases were separated in a separatory funnel.

Then, the total yield for the extraction step was calculated as the sum of products of BAC concentration in each phase and the volume of each phase relative to the flavonoid content in the raw material.

Extracts were evaluated for flavonoid and chlorophyll content [6]. Chlorophyll, being a lipophilic compound, is a convenient marker for evaluating the degree of extraction of the lipophilic fraction from the raw material.

The ratios of solvents used by us were established beforehand as those providing the most complete extraction of the flavonoids.

The results indicate that using biphasic extractant systems could increase the yield of both hydrophilic (flavonoids) and lipophilic (chlorophylls) compounds compared with aqueous alcohol extractants. Our results showed that biphasic system 2, which contained CH\textsubscript{2}Cl\textsubscript{2}, gave the highest yield. The yield of flavonoids increased by 1.7 times;