Isoflavones in Different Parts of Common *Trifolium* Species

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An HPLC method was used to separate and determine four isoflavone compounds (genistein, daidzein, formononetin, and biochanin A) from different parts (stem, leaf, and flower) of seven common *Trifolium* species. In general, low isoflavone concentrations were found in *T. montanum*, *T. fragiferum*, *T. incarnatum*, and *T. repens*, while *T. alpestre*, *T. pratense*, and *T. subterraneum* contained higher amounts of these compounds. Each species and each plant organ have, according to our data, a different distribution of the examined isoflavone compounds. In the flower is the highest amount of genistein (*T. subterraneum*) daidzein (*T. pratense*), and biochanin A (*T. alpestre*). The leaf fractions showed a similar situation in the case of *T. subterraneum* and *T. alpestre* but not in the case of *T. pratense*. The stem fractions have in general the lowest total isoflavone concentrations and are rich in daidzein (*T. pratense*) or in daidzein and biochanin A (*T. subterraneum* and *T. alpestre*).

Keywords: *Isoflavones; Trifolium species*

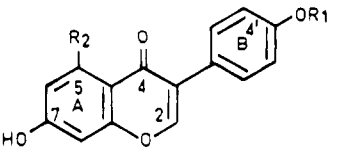
INTRODUCTION

The isoflavones of plants (for the chemical structures, see Figure 1) have a distinct taxonomical distribution because their occurrence is bound mainly to the family Fabaceae. The first phytochemical and physiological studies (Beck, 1964; Wong, 1962)—mainly in Australia—included the isolation, separation, and identification of these compounds. The qualitative and quantitative data of these first works were more or less problematical, and the data were uncomparable. Most analyses were made on *Trifolium subterraneum* (subterranean clover) (Rossiter and Beck, 1966a–c, 1967) on the effect of different ecological factors (temperature, light, and phosphate supply) or of ontogenic changes in isoflavone concentration. Other works were performed with *Trifolium alexandrinum* (Galvano et al., 1976; Shehata et al., 1982). The European common species (or different cultivated varieties) were and are very little investigated (primarily *Trifolium pratense*: Francis and Mukhina, 1981; Gosden and Jones, 1978; Wong, 1962). The metabolism and effect of plant estrogens in animals were examined by certain authors (Müller et al., 1989; Roshal and Tsinovyi, 1990) and summarized, too (Vetter, 1991). The main effects of these compounds in sheep are limited to the reproductive area, the more important effects being, from the point of view of animal production, temporary or permanent sterility, abortions, neonatal mortality, dystocias, and uterine prolapses. The aim of this work was to (1) compare four isoflavone compounds in seven common clover species and (2) compare these compounds in different organs of the plants (stem, leaf, and flower).

EXPERIMENTAL PROCEDURES

All plant materials were gathered in the Botanical Garden of Soroksár (Budapest) in the flowering phase. Thus, the soil and other ecological factors were in this case identical and the chemical differences are due only to other factors. The leaves, stems, and flowers of the gathered plants were separated, and the other operations were performed from the separate plant samples.

In our pre-examination was used a fluorometric method of Gosden and Jones (1978) to determine the formononetin content. The results were irreal great and changing; thus, in our opinion this method is unsuitable to real and precise isoflavone determination. Therefore, an HPLC method was used [based on the method of Patroni et al. (1982)], as follows:



	R_1	R_2
Genistein	H	OH
Biochanin A	CH_3	OH
Daidzein	H	H
Formononetin	CH_3	H

Figure 1. Chemical structure of the analyzed isoflavones.

The gathered, dried, and ground plant material (100–100 mg) was extracted (in methanol, at 55 °C, 30 min, in a shaker) and filtered. The extraction conditions are modified from those of Patroni et al. because they used a 10 min extraction at 60 °C. We worked with carefully dried (maximum temperature was 40 °C) plant material, although according to Jones (1979) no significant losses were registered in the formononetin content of red clover after a drying at 60 °C (!) for 12 h. From the above-mentioned samples were performed the determinations. The machine used was an HPLC chromatograph (Labor MIM, Hungary); the standing phase was Hypersil ODS, 5 μm, 250 × 4.6 mm, and the moving phase contained 55% methanol and 45% KH_2PO_4 solution (1%). The pH value of this was 4.0 (regulated with concentrated H_3PO_4). The velocity of the moving phase was 1.00 cm^3/min, and the wavelength of detection was 264 nm. The injected material was 50 mm^3. The percentage recovery of isoflavones in this method, according to our experiments, is higher than 90%. The determinations of daidzein, genistein, formononetin, and biochanin A were performed with suitable standard compounds, on the basis of retention times [according to basic works of Banwart et al. (1985), where all identifications were made only on the basis of retention times]. All determinations were performed in triplicate; the table contains the arithmetical mean and the standard deviation (SD).

RESULTS AND DISCUSSION

The concentrations of four investigated isoflavone compounds of plant parts from *Trifolium* species are summarized in Table 1. It was established that *T.*

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Table 1. Isoflavone Contents [Milligrams per Gram of Dry Weight, Arithmetic Means and Standard Deviations (SD) of the Analytical Data Are Given; Number of Replicates, 3] in Different Parts of the Examined *Trifolium* Species

species	part of plant	isoflavones				
		daidzein	genistein	formononetin	biochanin A	total
T. alpestre	leaf	0.332	0.059	0.020	1.530	1.941
		SD 0.008	0.005	0.004	0.011	
	stem	0.256	0.005	0.007	0.284	0.552
		SD 0.009	0.001	0.001	0.004	
	flower	0.217	0.006	0.079	0.964	1.266
		SD 0.005	0.002	0.005	0.012	
T. fragiferum	leaf	0.016	0.010	0.042	0.010	0.078
		SD 0.001	0.002	0.002	0.001	
	stem	0.008	0.028	0.112	0.010	0.158
		SD 0.001	0.004	0.007	0.001	
	flower	0.589	0.005	0.005	0.022	0.010
		SD 0.015	0.001	0.001	0.003	
T. incarnatum	leaf	0.018	0.094	0.352	0.010	0.474
		SD 0.005	0.002	0.006	0.002	
	stem	0.046	0.059	0.106	0.010	0.221
		SD 0.005	0.004	0.007	0.002	
	flower	0.017	0.025	0.112	0.010	0.164
		SD 0.004	0.002	0.011	0.002	
T. montanum	leaf	0.026	0.005	0.007	0.007	0.010
		SD 0.003	0.001	0.001	0.001	
	stem	0.002	0.003	0.004	0.004	0.005
		SD 0.001	0.001	0.001	0.001	
	flower	0.302	0.010	0.022	0.010	0.344
		SD 0.007	0.002	0.003	0.001	
T. pratense	leaf	0.349	0.023	0.377	0.318	1.067
		SD 0.005	0.003	0.008	0.010	
	stem	0.334	0.062	0.280	0.068	0.744
		SD 0.014	0.004	0.009	0.004	
	flower	0.461	0.019	0.391	0.338	1.209
		SD 0.019	0.003	0.011	0.010	
T. repens	leaf	0.005	0.005	0.007	0.010	0.027
		SD 0.001	0.001	0.002	0.001	
	stem	0.009	0.020	0.085	0.005	0.119
		SD 0.003	0.004	0.004	0.001	
	flower	0.006	0.023	0.052	0.013	0.094
		SD 0.001	0.004	0.001	0.001	
T. subterraneum	leaf	0.274	0.436	0.117	0.409	1.236
		SD 0.018	0.025	0.009	0.024	
	stem	0.220	0.051	0.094	0.183	0.548
		SD 0.020	0.003	0.007	0.005	
	flower	0.473	0.760	0.255	0.385	1.873
		SD 0.019	0.034	0.027	0.017	

montanum, *T. incarnatum*, *T. fragiferum*, and *T. repens* samples contain very small quantities of these compounds. The low isoflavone content of *T. repens* is identical with results of Saba et al. (1974). They published higher isoflavone contents only in the case of certain infected plants. The species *T. alpestre*, *T. subterraneum*, and *T. pratense* showed, however, significantly higher isoflavone content (total isoflavone concentrations in leaves: 1.94, 1.23, and 1.06 mg/g of dry weight, respectively). The flowers and the stems showed another order of range; thus, the different plant parts have other total isoflavone content. With regard to the rate and distribution of different flavonoid components, we see other compositions in the different species: the biochanin A content of *T. alpestre* is considerable, but the other two species contain only an insignificant quantity. From a phytochemical point of view it should be established that great differences (qualitative and quantitative) were determined in the *Trifolium* genus. The earlier methods (separation and determination techniques) did not give detailed and precise information or the investigations were made on only one or two species (mainly *T. subterraneum* and, rarely, *T. pratense*). The results of Rossiter (1970) (in Australia) were very important in the selection and breeding of new varieties of low (or lower) isoflavone concentrations. Of the few European investigations, the review of Rolinski (1969) was important, but we cannot evaluate the isoflavone contents of the plants because of the incompatibility of the analytical methods used. Our data have chemotaxonomical character and importance and can be useful to the breeding work of new *Trifolium* varieties with low (or lower) isoflavone concentrations.

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