JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Concentration of Isoflavones and Other Phenolics in the Aerial Parts of *Trifolium* Species

WIESLAW OLESZEK,* ANNA STOCHMAL, AND BOGDAN JANDA

Department of Biochemistry, Institute of Soil Science and Plant Cultivation, State Research Institute, ul. Czartoryskich 8, 24-100 Pulawy, Poland

Some species of the genus *Trifolium* are well-known for their content of isoflavones, which are natural compounds showing health-promoting activities. Until recently, only a few species of this genus have been characterized with respect to their composition. In the present study, 57 *Trifolium* species have been analyzed for their contents of isoflavones, phenolic acids, flavonoids, and clovamides. The cluster analysis of experimental data allowed us to identify a number of species, which should be of interest as potential sources of these metabolites. The isoflavone contents of the three species (*T. heldreichianum*, *T. scabrum*, and *T. subterraneum*) had extremely high amounts of these compounds, reaching 7–9% of dry matter, and the concentration in a number of other species was higher or at least comparable to the amounts occurring in *T. pratense*, one of the major isoflavone sources for the nutraceutical industry. Several species contained high amounts of all four analyzed groups of phenolics (isoflavones, phenolic acids, flavonoids, and clovamides). These species may also be of great interest as the association of several groups of active molecules is highly desired for effective disease prevention.

KEYWORDS: *Trifolium*; isoflavones; phenolic acids; flavonoids; clovamides; UPLC

INTRODUCTION

The genus *Trifolium* (Papilionoidae-Trifolieae) includes over 250 species. The majority of these species have not been phytochemically characterized (*1*). Only the species with agricultural significance such as *T. pratense* L., *T. repens* L., *T. resupinatum* L., *T. incarnatum* L., *T. hybridum* L., *T. pannonicum* L., *T. subterraneum* L., *T. fragiferum* L., and *T. medium* L. have been studied for the occurrence of saponins, cyanogenic glycosides, and phenolics (*1–4*). Some of these phytochemicals, such as cyanogenic glucosides, have been considered as antinutritional factors, lowering pasture quality. Another question that was connected to clover phytochemicals was the sheep fertility problems in Australia known as "clover disease" (*5*). In-depth studies showed that isoflavonoids present in clover (*T. subterraneum*) aerial parts with strong oestrogenic activity were the responsible factor.

This estrogenic activity of isoflavonoids is useful in clinical human nutrition. Epidemiological studies have shown a lower incidence of coronary heart disease and hormone-dependent cancers (breast and prostate) in Asian countries than in Western countries, at least in part correlated with the consumption of isoflavonoids present in dietary soy (*6, 7*), which are structurally similar to the estrogen 17β-estradiol (*8*). Recent studies confirmed that substituting soy beans for nonsoy protein in a therapeutic lifestyle diet improved the blood pressure and low-density lipoprotein cholesterol levels in hypertensive women and the blood pressure in normotensive postmenopausal women (*9*). These findings also suggested that the isoflavone content accounted for the observed effects.

Beneficial effects of isoflavones resulted in the introduction of dietary supplements fortified with these compounds into the U.S. and European markets. The only sources of isoflavones to be used for fortification are soybean and red clover. Thus, there is a demand for new sources of these compounds. Recently, Polasek and co-workers (*10*) published the results on two clover species, *T. pallescens* and *T. alpinum*, as promising sources of isoflavones. For the same reason, we determined concentrations of four major groups of phenolics, phenolic acids, clovamids, flavonoids, and isoflavones, in 57 *Trifolium* species. The seeds of the same collection of species were previously studied for the presence of saponins and flavonoids (*11*).

MATERIALS AND METHODS

Plant Material. Seeds of authenticated materials of 57 species of *Trifolium* were obtained from Genebank, Zentralinstitute für Pflanzengenetik und Kulturpflanzenforschung (Gatersleben, Germany). The origins of the species and their herbarium voucher numbers are listed in **Table 1**. Plants were cultivated (1 m × 1 m plots) in an experimental field of the Institute of Soil Science and Plant Cultivation in Pulawy, Poland. They were harvested at the beginning of flowering, lyophilized, finely powdered, and used for the successive extraction.

Extraction and Purification. The dried and finely powdered *Trifolium* tops (400 mg) were extracted with 40 mL of 50% MeOH at 100 °C in an ASE200 accelerated solvent extractor (Dionex, Sunnyvale, CA) at a working pressure of 1500 psi. Ten milliliters of these extracts was taken, and the solvent was removed under reduced pressure and

* To whom correspondence should be addressed. Tel: 48-81-8863421. Fax: 48-81-8864547. E-mail: wo@iung.pulawy.pl.

10.1021/jf072024w CCC: $37.00
Published on Web 09/12/2007

Table 1. List of Taxa Examined of the Genus *Trifolium*

no.	species (subspecies, variety)	origin	herbarium voucher
44	*T. alexandrinum* Jusl.	India	TRIF 30/79
20	*T. alpestre* L.	unknown	TRIF 201/93
31	*T. ambiguum* M. Bieb.	unknown	TRIF 178/75
5	*T. angustifolium* L.	France	TRIF 139/78
6	*T. apertum* Bobr.	unknown	TRIF 44/83
29	*T. arvense* L.	unknown	TRIF 40/78
36	*T. bocconei* Savi	Portugal	TRIF 81/79
49	*T. campestre* Schreb.	Sweden	TRIF 99/91
50	*T. carmeli* Boiss.	Israel	TRIF 100/81
37	*T. cernuum* Brot.	Portugal	TRIF 52/96
27	*T. cherleri* Jusl.	unknown	TRIF 74/96
10	*T. clypeatum* L.	Israel	TRIF 129/96
4	*T. curvisepalum* Tackh.	unknown	TRIF 53/94
47	*T. desvauxii* et Blume	United States	TRIF 143/82
43	*T. dubium* Sibth.	Germany	TRIF 103/79
55	*T. echinatum* M. Bieb. ssp. *supinu* (Savi) Aschers. Et Graebn.	Romania	TRIF 104/95
54	*T. fragiferum* L. ssp. *bonanni* (Presl) Soj.	Hungary	TRIF 208/79
33	*T. fragiferum* L. ssp. *fragiferum*	unknown	TRIF 37/83
57	*T. glomeratum* L.	Morocco	TRIF 107/81
16	*T. heldreichianum* Hausskn	unknown	TRIF 149/92
19	*T. hirtum* All.	unknown	TRIF 213/78
13	*T. hybridum* L.	unknown	TRIF 6/82
56	*T. incarnatum* L.	Czech Republic	TRIF 82/83
8	*T. isodon* Murb.	Denmark	TRIF 133/79
35	*T. isthmocarpum* Brot.	Portugal	TRIF 77/91
1	*T. lappaceum* L.	unknown	TRIF 55/83
7	*T. leucanthum* M. Bieb.	Israel	TRIF 131/77
40	*T. ligusticum* Balb.	Portugal	TRIF 113/83
25	*T. medium* Grufb. var. *medium*	unknown	TRIF 35/83
17	*T. medium* Grufb. var. *sarosiense* (Hazsl)	unknown	TRIF 179/83
28	*T. michelianum* Savi s l.	unknown	TRIF 79/81
52	*T. michelianum* Savi s. l. ssp. *balansae* (Boiss.)Thell	Bulgaria	TRIF 145/76
34	*T. miegeanum* Maire	Portugal	TRIF 116/79
32	*T. montanum* L.	unknown	TRIF 147/95
11	*T. nigrescens* Viv. ssp. *nigrescens*	Portugal	TRIF 117/96
46	*T. occidentale* D.E. Coombe	France	TRIF 255/91
51	*T. ochroleucon* Huds.	Slovakia	TRIF 173/96
42	*T. palidum* Waldst. et Kit.	Italy	TRIF 253/95
24	*T. pannonicum* Jacq.	unknown	TRIF 8/91
14	*T. phleoides* Pourr.	unknown	TRIF 132/94
41	*T. pratense* L.	Turkey	TRIF 186/75
48	*T. pratense* L. ssp. *expansum* (Waldst. et Kit) Simk)	Turkey	TRIF 188/83
15	*T. pratense* L. ssp. *sativum* (Schreb) Schübl. et Mart	unknown	TRIF 171/79
18	*T. pratense* L. ssp. *sativum* (Schreb) Schübl. et Mart. f. *albiflorum* Alef.	unknown	TRIF 151/78
23	*T. repens* L.	unknown	TRIF 15/79
3	*T. resupinatum* L. var. *majus* Boiss.	Iran	TRIF 61/83
21	*T. resupinatum* L. var. *minus* Boiss.	unknown	TRIF 57/83
12	*T. resupinatum* L. var. *resupinatum*	unknown	TRIF 43/80
26	*T. rubens* L.	unknown	TRIF 32/82
39	*T. scabrum* L.	Portugal	TRIF 120/79
22	*T. spumosum* L.	unknown	TRIF 67/83
9	*T. squarrosum* L.	unknown	TRIF 122/79
53	*T. stellatum* L.	Croatia	TRIF 215/94
45	*T. striatum* L.	France	TRIF 70/96
2	*T. subterraneum* L. ssp. *subterraneum*	United States	TRIF 259/91
38	*T. tomentosum* L.	Portugal	TRIF 218/79
30	*T. xerocephalum* Fenzl.	unknown	TRIF 80/75

temperature (50 °C). The crude extract was suspended in water (10 mL) and was passed through a C_{18} Sep-Pak (360 mg, 55–105 μm) cartridge (Waters Associates, Milford, MA) preconditioned with water. The cartridge was washed first with water (10 mL) to remove sugars and then with 70% MeOH (10 mL) to elute phenolics. This fraction was evaporated and redissolved in MeOH (10 mL) for analyses. Three independent extraction and purification procedures were performed for each species, and data were presented as mean values with standard deviations (**Table 2**).

Ultra-Performance Liquid Chromatography (UPLC) Analysis. The Acquity ultra-performance liquid chromatograph (Waters) consisting of binary solvent manager, sample manager, photodiode array detector (PDA), and Empower Pro 2.0 software was used. The profiling was performed on a 50 mm × 2.1 mm i.d., 1.7 μm, UPLC BEH C_{18} column (Waters) utilizing a gradient elution profile and a mobile phase consisting of 0.1% acetic acid in water (solvent A) and 40% MeCN (solvent B). The shape of the gradient used was as follows: 0 min, 100% A; 1 min, 90% A; 7 min, 35% A; 7.5 min, 100% B; 9.5 min, 100% B; and 10 min, 100% A. The column was maintained at 50 °C, and the flow rate was kept constant at 0.35 mL/min. The injection volume was 5 μL. The UPLC profiles were integrated at 254 nm, and the shape of the absorption spectrum of each peak was observed with a PDA. The peak areas of the same shape were selected, and the concentration was determined for each of them separately based on calibration curves of chlorogenic acid for phenolic acids and clovamides, quercetin glucoside for flavonoids, and daidzin for isoflavones.

Statistical Analysis. The statistics and cluster analysis (*K*-means method) were performed with the Stagraphics program.

Table 2. Concentration of Total Phenolics, Phenolic Acids (Equivalent of Chlorgenic Acid), Clovamides (Equivalent of Chlorogenic Acid), Flavonoids (Equivalent of Quercetin Glucoside), and Isoflavones (Equivalent of Daidzin) in 57 *Trifolium* Species

	mg/g dm ± SD				
species	phenolic acid	clovamide	flavonoids	isoflavones	total phenolics
cluster C1					
1	4.61 ± 0.18	ND [a]	6.68 ± 0.47	58.13 ± 1.03	69.42
14	3.10 ± 0.08	ND	9.07 ± 0.27	45.23 ± 0.44	57.40
19	7.30 ± 0.50	ND	2.50 ± 0.03	23.79 ± 0.16	34.22
20	6.89 ± 0.10	ND	8.45 ± 0.30	35.21 ± 0.16	50.55
25	11.50 ± 0.40	3.06 ± 0.01	8.68 ± 0.26	62.37 ± 0.15	85.61
2	5.48 ± 0.40	ND	7.04 ± 0.17	82.98 ± 0.38	95.50
16	ND	ND	9.21 ± 0.53	88.38 ± 0.22	97.59
39	0.66 ± 0.00	ND	7.67 ± 0.17	72.76 ± 0.43	80.93
cluster C2					
5	ND	ND	23.23 ± 0.33	3.16 ± 0.00	26.39
36	1.06 ± 0.01	ND	22.71 ± 0.02	18.70 ± 0.03	42.47
11	0.28 ± 0.00	ND	15.88 ± 0.28	ND	16.16
24	1.11 ± 0.01	0.36 ± 0.00	15.74 ± 0.39	ND	17.21
47	ND	ND	19.08 ± 0.21	2.20 ± 0.00	21.28
7	ND	ND	32.40 ± 0.68	12.55 ± 0.02	44.95
cluster C3					
3	4.42 ± 0.02	ND	12.83 ± 0.12	ND	17.25
29	0.63 ± 0.00	ND	9.80 ± 0.02	8.58 ± 0.01	19.01
53	ND	ND	8.31 ± 0.01	1.50 ± 0.01	9.81
56	ND	ND	8.51 ± 0.01	ND	8.51
30	4.13 ± 0.04	ND	3.81 ± 0.03	ND	7.94
35	5.38 ± 0.05	ND	4.00 ± 0.02	ND	9.38
57	4.76 ± 0.04	ND	4.76 ± 0.03	ND	9.52
46	5.21 ± 0.09	0.20 ± 0.00	2.09 ± 0.01	ND	7.50
48	5.82 ± 0.06	1.22 ± 0.03	2.05 ± 0.12	10.39 ± 0.14	19.48
31	6.37 ± 0.06	ND	6.06 ± 0.03	0.62 ± 0.00	13.05
50	6.24 ± 0.38	ND	7.75 ± 0.03	1.65 ± 0.01	15.64
33	5.17 ± 0.02	ND	6.19 ± 0.01	4.23 ± 0.02	15.59
43	0.42 ± 0.10	0.47 ± 0.12	3.69 ± 0.66	ND	4.58
45	1.09 ± 0.05	ND	4.67 ± 0.12	ND	5.76
54	0.62 ± 0.14	ND	5.18 ± 0.21	5.50 ± 0.12	11.30
49	1.19 ± 0.18	0.54 ± 0.11	2.11 ± 0.03	ND	3.84
cluster C4					
4	10.28 ± 0.29	ND	8.35 ± 0.02	2.82 ± 0.20	21.45
40	11.93 ± 0.44	ND	10.64 ± 0.20	ND	22.57
51	9.94 ± 0.02	2.46 ± 0.01	10.11 ± 0.20	1.10 ± 0.10	23.61
12	9.04 ± 0.25	ND	5.54 ± 0.69	ND	14.58
37	8.40 ± 0.07	ND	5.85 ± 0.04	ND	14.25
13	9.58 ± 0.37	0.44 ± 0.01	5.22 ± 0.68	ND	15.24
17	11.65 ± 0.80	ND	5.54 ± 0.01	1.58 ± 0.08	18.77
21	12.21 ± 0.24	0.15 ± 0.02	5.49 ± 0.20	1.62 ± 0.04	19.47
26	12.15 ± 0.13	ND	7.87 ± 0.10	ND	20.02
23	12.20 ± 0.72	1.13 ± 0.10	2.33 ± 0.03	ND	15.66
27	10.45 ± 0.10	ND	3.64 ± 0.18	5.53 ± 0.14	19.62
38	10.02 ± 0.19	ND	3.52 ± 0.01	ND	13.54
28	9.87 ± 0.04	ND	1.44 ± 0.05	ND	11.31
52	9.12 ± 0.38	ND	1.42 ± 0.10	0.65 ± 0.01	11.19
22	18.08 ± 0.25	0.64 ± 0.01	0.93 ± 0.07	ND	19.65
32	15.14 ± 0.72	ND	12.03 ± 0.20	ND	27.17
cluster C5					
6	8.83 ± 0.13	4.38 ± 0.02	9.16 ± 0.10	4.90 ± 0.05	27.27
44	7.53 ± 0.02	5.84 ± 0.13	10.54 ± 0.51	3.20 ± 0.02	27.11
10	9.31 ± 0.34	7.75 ± 0.18	10.30 ± 0.12	ND	27.36
9	8.23 ± 0.21	7.83 ± 0.02	15.56 ± 0.18	20.50 ± 0.22	51.77
55	5.43 ± 0.02	4.27 ± 0.18	15.40 ± 0.32	6.47 ± 0.13	31.57
15	14.07 ± 0.18	7.91 ± 0.50	3.55 ± 0.14	14.80 ± 0.82	40.33
18	12.80 ± 0.92	4.87 ± 0.32	2.51 ± 0.01	11.80 ± 0.08	31.98
34	8.05 ± 0.81	6.98 ± 0.20	7.63 ± 0.25	26.49 ± 0.28	49.15
41	10.06 ± 0.43	4.27 ± 0.04	4.79 ± 0.12	39.51 ± 0.98	58.63
8	12.86 ± 0.22	9.90 ± 0.38	8.48 ± 0.12	23.53 ± 0.45	54.77
42	14.20 ± 0.55	12.94 ± 0.15	0.61 ± 0.00	7.31 ± 0.52	35.06

[a] ND, not detected.

RESULTS AND DISCUSSION

The analyses of UPLC profiles of 57 *Trifolium* species with PDA detection clearly indicated that they contain four distinct groups of phenolics that can be identified based on their absorption spectra (**Figure 1**). The first group showed absorption spectra with the maximum absorption at 245 and 320 nm, identical to the spectrum of chlorogenic acid. The second group possessed spectra with two absorption maxima at 291 and 319

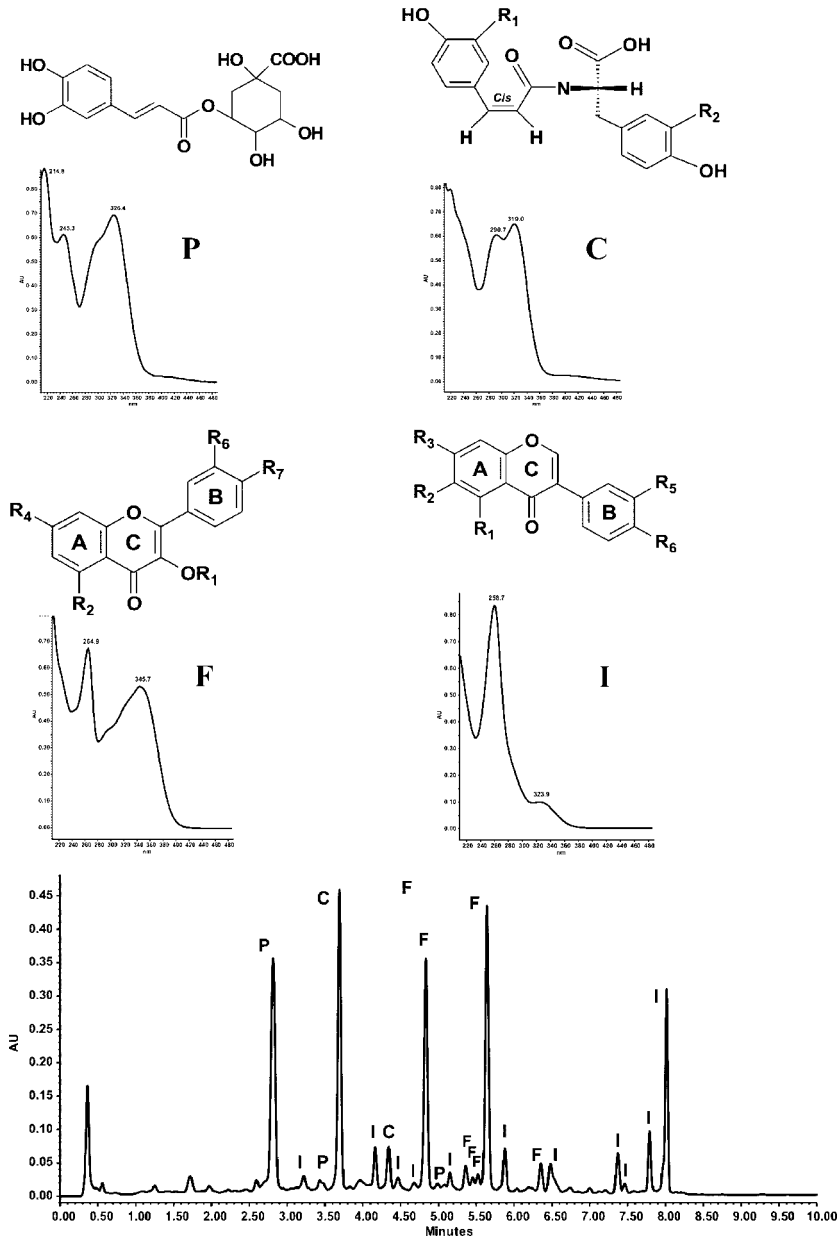

Figure 1. Characteristic absorption spectra of the four groups of phenolics identified in *Trifolium* species and UPLC profile of *T. squarrosum* (**9**); P, phenolic acid; C, clovamid; F, flavonoid; and I, isoflavone.

nm, identical to those reported previously (*10*) as characteristic for clovamides. Only two compounds of this structure could be monitored in some species, and they corresponded to *trans*- and *cis*-clovamide. The third group had two absorption maxima and overall spectral shapes characteristic of flavonoids. The fourth group contained only one maximum with a small shoulder, and this was characteristic for isoflavones. No other phenolics were present in any of the analyzed species.

The four groups were quantified using appropriate standards with UPLC. The total quantity was calculated for each peak of the same absorption profile, and the quantities were summarized as presented in **Table 2**.

The cluster analysis of quantification data allowed us to distinguish several major groups of species differing in phenolic composition and quantity (**Figure 2**). The first cluster (C1), containing *T. lappaceum*, *T. phleoides*, *T. hirtum*, *T. alpestre*, *T. medium*, *T. subterraneum*, *T. heldreichianum*, and *T. scabrum*, was characterized by high concentrations of isoflavones, ranging from 51 to 97 mg/g of dry matter. The highest amount, reaching nearly 10% of dry matter, was found in *T. scabrum*, *T. subterraneum*, and *T. heldreichianum*. All of the species belonging to this cluster containing from 2.5 to 9.2 mg/g of dry matter of flavonoids showed a lack of clovamides (except for *T. medium*) and contained different amounts of phenolic acids, ranging from 0 to 11.5 mg/g of dry matter.

The common feature of the second cluster (C2), which included *T. angustifolium*, *T. bocconei*, *T. nigrescens*, *T. pannonicum*, *T. desvauxii*, and *T. leucanthum*, was a high concentration of flavonoids ranging from about 16 to 32 mg/g of dry matter. The species of this cluster contained trace amounts of phenolic acids, and they lacked the clovamides. Their isoflavone concentration was also low, excluding *T. bocconei* and *T. leucanthum*, where the amount of these compounds exceeded 1% of dry matter.

The third cluster (C3), which included *T. resupinatum* var. *majus*, *T. arvense*, *T. stellatum*, *T. incarnatum*, *T. xerocephalum*, *T. isthmocarpum*, *T. glomeratum*, *T. occidentale*, *T. pretense* ssp. *expansum*, *T. ambiguum*, *T. carmeli*, *T. fragiferum* ssp.

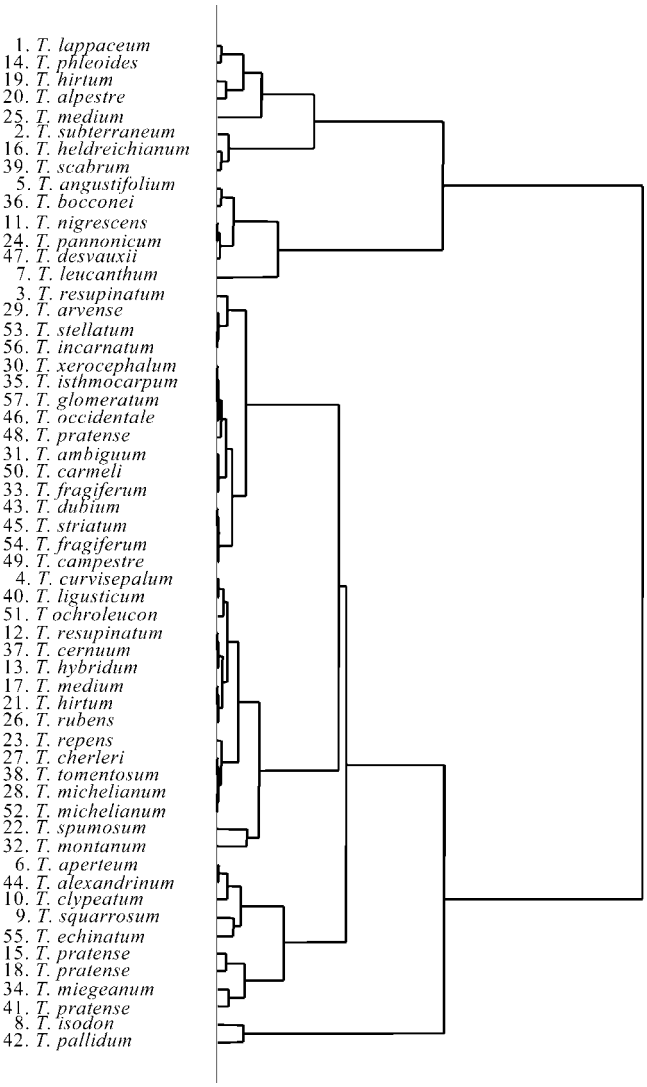

Figure 2. Cluster analysis of the concentration of phenolics in *Trifolium* species.

fragiferum, *T. dubium*, *T. striatum*, *T. fragiferum* ssp. *bonnani*, and *T. campestre*, showed the lowest phenolic content. Almost all of the species of this group did not contain clovamides. Most of them were free of isoflavones, or their content was very low. The exception was *T. pretense* ssp. *expansum* in which the isoflavone content was around 1% of dry matter. The contents of phenolic acids and flavonoids in all of these species were also relatively low.

The fourth cluster (C4), which included *T. ligusticum*, *T. ochroleucon*, *T. resupinatum* var. *resupinatum*, *T. cernuum*, *T. hybridum*, *T. medium* var. *sarosiense*, *T. hirtum*, *T. rubens*, *T. repens*, *T. cherleri*, *T. tomentosum*, *T. michelianum* Savi s l., *T. michelianum* ssp. *balansae*, *T. spumosum*, and *T. montanum*, was similar to the cluster C3 in the concentration of isoflavones, flavonoids, and cloveamides. The feature that distinguished C3 and C4 was a high concentration of phenolic acids in C4, which for most species ranged around 1–1.8% of dry matter.

The last, the fifth cluster (C5), contained 11 species including *T. aperteum*, *T. alexandrinum*, *T. clypeatum*, *T. squarrosum*, *T. echinatum*, *T. pretense* ssp. *sativum*, *T. pretense* ssp. *sativum* f. *albiflorum*, *T. miegeanum*, *T. pretense*, *T. isodon*, and *T. pallidum*. The common feature of this cluster was a relatively high concentration of all four groups of phenolics studied. They all contained clovamides, the concentration of which ranged from 4 to 13 mg/g of dry matter, but most of the species of this group also had a high concentration of phenolic acids, flavonoids, and in some cases isoflavones.

Isoflavones have attracted attention due to their role in the amelioration of postmenopausal symptoms, the activities related to cardiovascular diseases, cognitive function, and breast and prostate cancers (*12*). Isoflavone-based nutraceuticals are the most assayed polyphenol supplements, and some representative intervention trials are being performed. The increasing interest in isoflavones as dietary components and their scarcity in Western diets as compared to the Asian diet, where they are abundant due to soy consumption, has resulted in increasing demand for new plant sources of these compounds. *T. pratense* is (besides soybean) one of the plant sources of these compounds.

In the present research, there were four different subspecies of *T. pretense* analysed. Three of them (species **15**, **18**, and **48**) had very similar concentrations of isoflavones ranging around 1% of dry matter. The species *T. pratense* originating from Turkey (**41**) contained about 4% of these compounds. This finding clearly indicates that for commercial purposes precise identification to the subspecies level is essential for the most effective yield of isoflavones.

Screening of *Trifolium* species for isoflavone concentration showed that there are a number of species with extremely high amounts of these compounds. From this point of view, the species grouped in clusters C1 and C5 are most interesting. Hypothetically, the most valuable species are *T. heldreichianum*, *T. scabrum*, and *T. subterraneum*, in which the isoflavone concentration ranged between 7 and 9% of dry matter. They can be recognized as very good commercial sources of these compounds, but nearly all of the species classified in clusters C1 and C5 contained comparable or higher isoflavone contents than *T. pratense*. They all should be recognized as potential commercial plant sources.

T. bocconei and *T. leucanthus*, which were classified in the less valuable cluster C2, may also be of interest. Their isoflavone content is comparable to that of *T. pratense*, but they both contain high amounts of flavonoids (2–3% of dry matter), which may also be of great interest due to different biological activities of these compounds (*13*).

The least interesting group from the point of view of potential commercial value seems to be those species classified in cluster C3, as all of the species contained rather low concentrations of total phenolics (below 2% of dry matter), mostly phenolic acids and flavonoids. Also, the species classified in cluster C4 were rather low in total phenolics with a dominant group of phenolic acids. Some of these could be of interest due to possible antioxidant activities of the phenolic acids.

Special attention should be paid to the species from the cluster C5. Some of these species contained 4–5% of total phenolics, comprising a mixture of all four groups analyzed. This is not a common feature in the plant kingdom. Most often, species contain one or two dominant groups of secondary metabolites. The presence of considerable quantities of four different phenolic groups may provide synergistic health effects in extracts from these plants. The association of several groups of active molecules, as naturally happens in some foods, might be more effective in disease prevention than single compounds (*14*). In particular, the presence of isoflavones has been restricted to very few taxonomic groups and the co-occurrence of a high content of other polyphenols may be a desired combination.

The vast research performed on the isoflavone activity indicates that derivatives of daidzein and genistein are structurally most related to the estrogen 17β-estradiol, and these are most desired compounds. More detailed research is needed to

determine the structural characteristics of phenolics, especially isoflavones, in *Trifolium* species identified in the present research as promising sources of these compounds.

ACKNOWLEDGMENT

We thank Prof. Dr. A. Graner of Genebank, Zentralinstitut für Pflanzengenetic und Kulturpflanzenforschung (Gatersleben, Germany), for providing us with authenticated *Trifolium* seeds and Ing. M. Gnieciak and B. Ciarkowska for technical assistance.

LITERATURE CITED

(1) Bisby, F. A.; Buckingham, J.; Harborne, J. B. *Phytochemical Dictionary of Leguminosae*; Chapman and Hall: London, 1994.
(2) Sakamoto, S.; Kfuji, S.; Kuroyanagi, M.; Ueno, A.; Sekita, S. Saponins from *Trifolium repens*. *Phytochemistry* **1992**, *31*, 1773–1777.
(3) Butler, G. W. The distribution of cyanoglucosides linamarin and lotaustralin in higher plants. *Phytochemistry* **1965**, *4*, 127–131.
(4) Stochmal, A.; Oleszek, W. The changes of cyanogenic glucosides in white clover (*Trifolium repens* L.) during the growing season. *J. Agric. Food Chem.* **1997**, *11*, 4333–4336.
(5) Underwood, E. J.; Shier, F. L. The performance of oestrogenic effects of subterraneum clover grazing on the ewe. *Aust. Vet. J.* **1951**, *27*, 61–67.
(6) Tham, D. M.; Gardner, C. D.; Haskel, W. L. Potential health benefits of dietary phytooestrogens: A review of the clinical, epidemiological, and mechanistic evidence. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 2223–2235.
(7) Beaglehole, R. International trends in coronary heart disease mortality, morbidity, and risk factors. *Epidemiol. Rev.* **1990**, *12*, 1–15.
(8) Vaya, J.; Tamir, S. The relation between the chemical structure of flavonoids and their oestrogen-like activities. *Curr. Med. Chem.* **2004**, *11*, 1333–1343.
(9) Welty, F. K.; Lee, K. S.; Lew, N. S.; Zhou, J.-R. Effect of soy nuts on blood pressure and lipid levels in hypertensive, and normotensive posmenopausal women. *Arch. Intern. Med.* **2007**, *167*, 1060–1067.
(10) Polasek, J.; Queiroz, E. F.; Hostettmann, K. On-line identification of phenolic compounds of *Trifolium* species using HPLC-UV-MS and post-column UV-derivatisation. *Phytochem. Anal.* **2007**, *18*, 13–23.
(11) Oleszek, W.; Stochmal, A. Triterpene saponins and flavonoids in the seeds of *Trifolium* species. *Phytochemistry* **2002**, *61*, 165–170.
(12) Verheus, M.; van Gils, C. H.; Keinan-Boker, L.; Grace, P. B.; Bingham, S. A.; Peeters, P. H. M. Plasma phytotoestrogen and subsequent breast cancer risk. *J. Clin. Oncol.* **2007**, *25*, 648–655.
(13) Rice-Evans, C. *Wake Up to Flavonoids*; The Royal Society of Medicine Press Ltd.: London, 2000.
(14) Russo, G. L. Ins and outs of dietary phytochemicals in cancer chemoprevention. *Biochem. Pharmacol.* **2007**, *74*, 533–544.

Received for review July 6, 2007. Revised manuscript received August 12, 2007. Accepted August 15, 2007. The work has been financed by the Polish Ministry of Science and Higher Education (Contract 579/E-178/SPB/COST/KN/DWM 53/2005–2007) and by European Community 6FP Project NUTRA-SNACKS (Contract 023044 FOOD).

JF072024W