JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isoflavone Profiles of Red Clovers and Their Distribution in Different Parts Harvested at Different Growing Stages

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The isoflavone compositions and concentrations in the leaf, flower, petiole, and stem of 13 red clover cultivars were studied using high-performance liquid chromatography coupled with a diode array and a mass spectrometric detector with negative electrospray ionization. Different cultivars showed significantly different concentrations of individual and total isoflavones. The leaf contained the highest overall concentration, followed by the stem, petiole, and flower. Biochanin A and formononetin were the predominant isoflavones in all cultivars and all parts, along with eight other minor aglycones, daidzein, genistein, glycitein, irilone, orobol, pratensein, pseudobaptigenin, and prunetin, and four minor malonylglycosides, genistein-7-glucoside-6″-malonate, orobol-7-glucoside-6″-malonate, formononetin-7-glucoside-6″-malonate, and biochanin A-7-glucoside-6″-malonate. The isoflavone compositions and concentrations were also found to be different between red clover parts harvested at the early bud stage and the late flowering stage. Sample storage and handling prior to analysis were also found to be important. Samples in this study were kept at −5 °C for a few days before being freeze-dried and were found to contain mainly the aglycones of isoflavones. This may actually be an advantage in that "natural" and more bioactive isoflavones can be obtained without using chemical hydrolysis. Findings in this study therefore provide important information for developing isoflavone-rich red clovers and for optimizing harvest timing and choosing the right part of the red clover plant.

KEYWORDS: Red clover; *Trifolium pratense* L.; isoflavone; HPLC; LC-MS; ESI-MS

INTRODUCTION

Soy bean and soy products are known as the richest food sources of isoflavones, a group of phytoestrogens, among which genistein and daidzein are most studied (*1*, *2*). However, recent research has shown that another leguminous plant, red clover (*Trifolium pratense* L.), albeit not frequently used as a food source, also contains genistein and daidzein, and more interestingly, it contains significantly higher concentrations of biochanin A and formononetin, methylated derivatives of genistein and daidzein, respectively (**Figure 1**). Red clover was originally used as a medicinal herb by the indigenous people of North America for whooping cough, gout, and cancer (*3*), and others traditionally have used it in the treatment of asthma, bronchitis, coughs, athlete's foot (*4*), and eczema and psoriasis (*5*). However, exploration on the potential application of red clover preparations for alternative hormone replacement therapy (HRT) as selective estrogen receptor modulators and in the management of menopause has only been attempted in recent years (*6*, *7*), due to highly publicized reports on the adverse effects of HRT, particularly in causing elevated coronary heart diseases and stroke (*8*) and dementia (*9*).

Red clover isoflavones have been extracted and commercialized as nutraceuticals (Promensil from Novogen) (Novogen, Australia). According to the company, isoflavones in their products were in natural aglycone forms, rather than in glycoside or malonylglycosides as found in soybean. However, the overwhelming majority of the literature indicates that the major forms of isoflavone in red clover were glycosides and malonylglycosides (*5*, *10*, *11*). Forms of isoflavones (aglycones vs glycosides) are important because they directly affect the bioavailability and thus the bioactivity of isoflavones. Most studies have shown that the malonate conjugates of isoflavones are not stable during processing (*12*−*14*). The glycosides of isoflavones, on the other hand, are relatively stable; however, they are not considered the biologically active form. Hydrolysis of the glycosides occurs during extraction under conditions that allow the enzyme β-glucosidase to be active (*10*, *11*). The glycosides can also be hydrolyzed biologically in animal guts or by bacteria isolated from human intestinal microflora (*15*). The hydrolysis products, i.e., the aglycones, were found to be

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10.1021/jf0614589 CCC: $33.50 Published 2006 by the American Chemical Society
Published on Web 07/19/2006

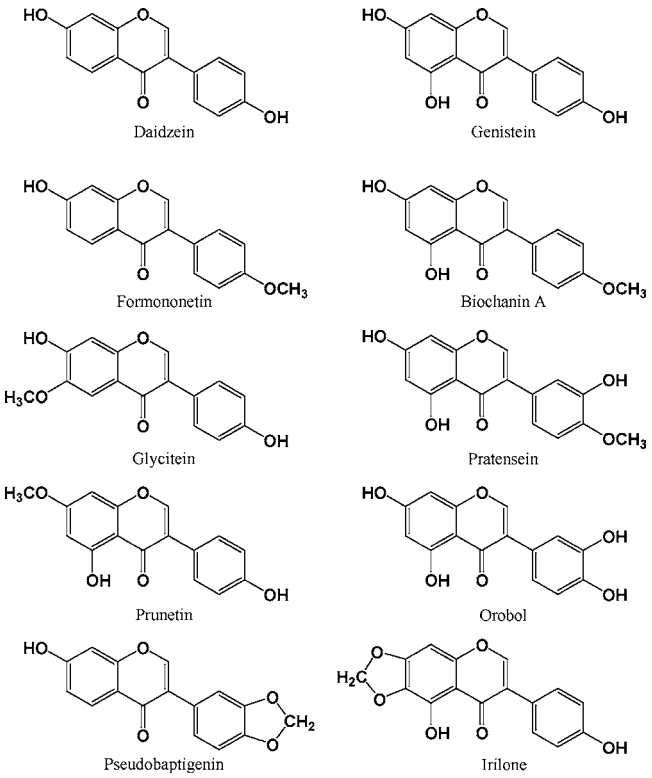

Figure 1. Isoflavone aglycones identified in red clovers.

absorbed at a more rapid rate and at higher levels as compared with the glycosides (*16*, *17*). Genistein and daidzein, but not their glycosides, are readily transported across human intestinal epithelial cell monolayers (*18*). The aglycones, for these reasons, are often considered the active form of the isoflavones. On the other hand, biochanin A and formononetin are found to be demethylated in the liver to form genistein and daidzein, respectively (*19*, *20*). Clinical and preclinical studies also found that the demethylation occurs in humans and animals (*21*, *22*), and the resulting daidzein and genistein may be absorbed or further metabolized to many specific metabolites including equol (*19*−*23*).

Isoflavone concentrations and profiles in red clovers are affected by many factors. Sivesind and Seguin (*24*) examined the effects of the environment, cultivar, plant maturity, plant part, and preservation method on the concentration of the two predominant isoflavones in red clover, formononetin and biochanin A. In a multiyear, multisite trial, the total isoflavone concentration in 10 cultivars ranged between 8.92 and 12.75 mg/g of dry matter (DM) averaged across sites, harvests, and years. They also found that leaves contained the highest concentration of isoflavones. Drying methods were found to critically affect the isoflavone contents in the leaf samples of red clover. It was found that nearly all intact isoflavones were in glucoside or malonylglucoside forms; freeze drying had the least impact on these native compounds, while vacuum drying had the most, resulting in total conversion to aglycones, mainly formononetin and biochanin A. It was considered that the slow thawing and drying process (several days at room temperature) during the vacuum drying allowed enzymatic conversion of 95% of the isoflavone glucosides to their aglycones (*10*). Aglycones were less than 1% of the total isoflavones in freeze-dried samples.

Different plant parts, and the same parts harvested at different growth periods, showed different concentrations of isoflavones. However, genetic variation seems to be a fundamental factor; hence, an active breeding program at Agriculture & Agri-Food Canada was established to obtain information on isoflavone contents in red clovers for the evaluation of this crop as a potential source of nutraceuticals and functional foods. In this paper, we report on the isoflavone profiles in 13 red clover cultivars and advanced breeding lines, including diploid and tetraploid cultivars, the distribution of these isoflavones in different parts of the plant (leaf, stem, petiole, and flower), and the effect of harvest timing. Instead of focusing on the major isoflavones, i.e., formononetin and biochanin A, we used high-performance liquid chromatography diode array detection mass spectrometry (HPLC-DAD-MS) for the identification and quantification of all detectable isoflavones.

MATERIALS AND METHODS

Chemicals and Solvents. Commercially available isoflavone standards including biochanin A, daidzein, formononetin, genistein, glycitein, and their corresponding glucosides and prunetin were purchased from Sigma-Aldrich Co. (Oakville, ON). Water used for HPLC analysis was purified in-house from distilled and deionized water using a NanoPure system (Dubuque, IA). All other solvents were of HPLC grade and were purchased from Caledon Laboratories Ltd. (Georgetown, ON).

Plant Materials and Sampling Method. Thirteen red clover populations, diploid and tetraploid, from a broad range of origins were sampled at various growth stages. Ten plants per replication from each population were grown in the greenhouse at the Crops and Livestock Research Centre (Charlottetown, PEI, Canada). Plants were harvested at two growth stages, the early bud stage (EB) when the buds were just visible, and the late flowering stage (LF) when the majority of buds had reached full flowering and some were beginning to mature (October 2003). Arial parts of five top-growing red clover plants from each genotype were cut, and the stems, petioles, leaves, and flowers (LF only) were separated. Tissues from the five plants of the same cultivar were pooled for each given sampling date. Fresh samples were immediately placed on ice and then transferred to a −5 °C freezer until freeze drying (<1 week). Samples were weighed and dried at −31 °C for 3 days using a freeze dryer (Hull, model #120FS175, Hull Corp., Hatboro, PA).

Sample Preparation. The freeze-dried samples were ground into powder before solvent extraction. Each sample (0.25 g) was extracted with 10 mL of 70% methanol at room temperature (22 °C) for 16 h, filtered through 0.45 μm Acrodisc syringe filter (Gelman Laboratory, MI), and stored at −20 °C until HPLC analysis. A parallel extraction of 0.25 g of sample with 10 mL of 70% methanol containing 2 M HCl was also carried out at 85 °C for 2 h for all samples. The recovery rates of the first extraction were >96%. A sequential extraction for 2 h only recovered <4%.

HPLC Conditions. An HPLC system (Agilent Technology 1100 Series, Palo Alto, CA) equipped with a quaternary pump, an inline degasser, a thermostatic autosampler, and a DAD was used for the identification and quantification of various isoflvones in the samples. A Phenomenex phenospheres ODS-2 BOA column (150 mm × 4.6 mm) with a C18 guard column (Torrance, CA) was used for the separation. The binary mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid (solvent B), and a gradient program was used as follows: 100 to 50% B in 40 min, 50 to 20% B in 5 min, 20 to 0% B in 5 min, and 0% B back to 100% B in 5 min. The flow rate was 1.0 mL/min for a total run time of 55 min. The injection volume was 10 μL for all standards and samples. All standards were dissolved in dimethyl sulfoxide/methanol (1:9, v/v). The detector was set at 260 nm for monitoring isoflavones.

LC-MS Conditions. LC-MS was performed using HPLC coupled to a photodiode array UV detector (Finnigan MAT Spectra System UV6000LP, San Jose, CA) equipped with a Finnigan LCQ Deca electrospray ionization mass spectrometer (HPLC-ESI-MS) operated in a negative ion mode. The same separation conditions as described in the HPLC analysis were used in the LC-MS. The instrument parameters were optimized against gallocatechin prior to sample

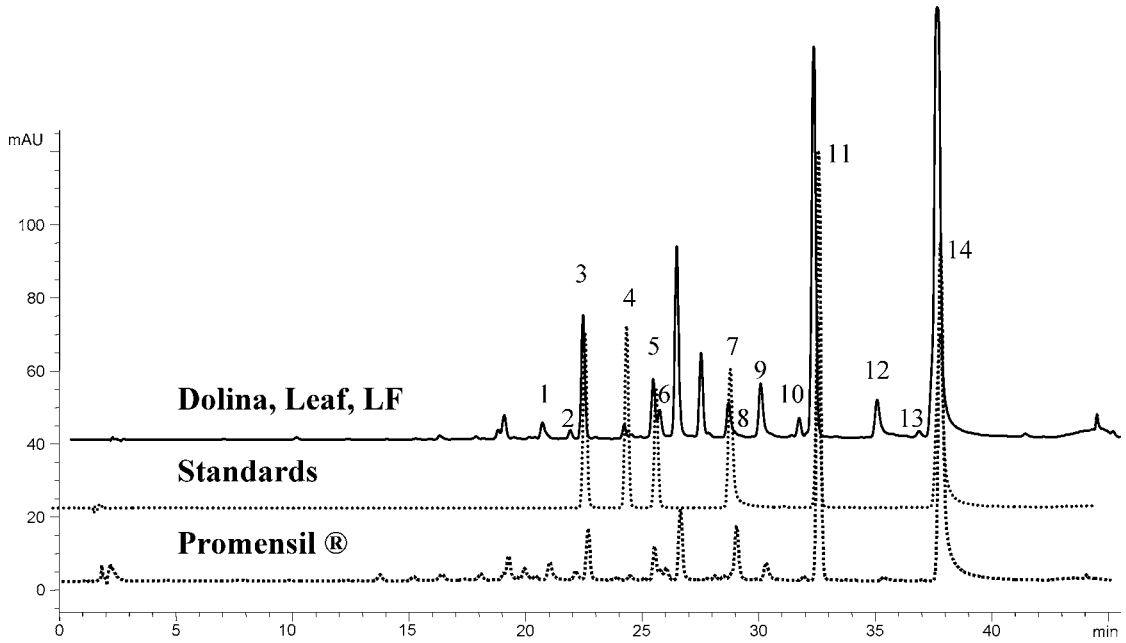

Figure 2. HPLC profiles of red clover leaf extract (cultivar Dolina, late flowering) and a commercial product Promensil. Refer to **Table 1** for peak identification.

Table 1. LC-DAD-ESI-MS Identification of Isoflavones in Red Clover Leaves

peak	t_R (min)	$[M-1]^-$ (*m*/*z*)	$[M+36-1]^-$	$[2M-1]^-$	$[2M+36-1]^-$	major MS fragment	λ_{max} (nm)	identification
1	21.24	517				269	249, 354	genistein-7-glucoside-6″-malonate
2	22.17	533	569			489	264, 342	orobol-7-glucoside-6″-malonate
3	24.42	253	289	507			249, 302	daidzein
4	24.82	285	321				261, 290	orobol
5	25.70	515	551	1031		267	250, 298sh[a]	formononetin-7-glucoside-6″-malonate
6	25.87	283	319	567		268	258	glycetein
7	28.90	269	539		575	307	260	genistein
8	29.20	531	567	1063		487	260	biochanin A-7-glucoside-6″-malonate
9	30.32	299	335	599	635	284	262, 284sh[a]	pratensein
10	31.83	281	317				250, 295	pseudobaptigenin
11	32.47	267		535		252	260, 324	formononetin
12[b]	35.30	283					270	prunetin
13[b]	37.02	297					260, 291	irilone
14	37.83	283	319			268	261, 326	biochanin A

[a] Shoulder. [b] Only molecular ions were detected under the mass spectrometry conditions used in this study.

analysis. Briefly, the shear gas and auxiliary flow rates were set at 48 and 34 (arbitrary unit), respectively. The capillary voltage was set at −14 kV, and its temperature was controlled at 250 °C. The entrance lens voltage was fixed at 64 V, and the multipole RF amplitude was set at 400 V. The ESI needle voltage was controlled at 6 kV. The tube lens offset was −30 V, the multipole lens 1 offset was 4.25 V, and the multipole lens 2 offset was 12.5 V. The electron multiplier voltage was set at −880 V for ion detection.

Identification and Quantification. Identification of isoflavones was achieved by comparing their retention times, UV/vis, and MS spectra with those of the standards. For those compounds without commercially available standards, the compounds were tentatively identified by using UV/vis and MS spectra. To compare our results to other previous studies, isoflavone content was quantified in the hydrolyzed samples. Six isoflavone standards including biochanin A, daidzein, formononetin, genistein, glycitein, and prunetin were used to obtain the standard curves of major isoflavones. The concentration of those isoflavones without standards was calculated by using the standard curve of biochanin A.

RESULTS AND DISCUSSION

Isoflavone Glycosides vs Aglycones. While glycosides and their malonylglycosides have been reported as the major forms of isoflavones in red clover, we found that in all samples, the overwhelming majority of isoflavones was in the aglycone form. Isoflavones in leaf samples were predominantly biochanin A and formononetin, along with eight other minor aglycones, daidzein, genistein, glycitein, irilone, orobol, pratensein, pseudobaptigenin, and prunetin, and four minor malonylglycosides, genistein-7-glucoside-6″-malonate, orobol-7-glucoside-6″-malonate, formononetin-7-glucoside-6″-malonate, and biochanin A-7-glucoside-6″-malonate (**Figure 2** and **Table 2**). In the stems, petioles, and flowers, it was the formononetin that predominated the profiles (**Tables 3**−**5**). These compounds were identified by congruent retention times, UV absorbance and MS with the standards, and the elution pattern reported by others (**Table 1**). Under the conditions used in this study, the ESI-MS in negative ion mode detected all molecular ions $[M-1]^-$, most of their adduct ions $[M+36-1]^-$, and some dimeric ions $[2M-1]^-$ and dimeric adduct ions $[2M+36-1]^-$ (**Table 1**). The aglycones were also confirmed by hydrolyzing the samples with HCl and comparing the profiles before and after the hydrolysis (**Figure 2**). As can be found, the major isofla-

Table 2. Isoflavone Concentrations (mg/g Dry Weight) in Leaves of the EB and the LF

	daidzein	orobol	glycitein	genistein	pratensein	pseudobaptigenin	formononetin	prunetin	irilone	biochanin A	grand total
						EB					
Amos	0.55 ± 0.35	ND	1.51 ± 0.18	0.62 ± 0.23	0.82 ± 0.04	0.16 ± 0.02	8.15 ± 3.14	0.43 ± 0.13	0.02 ± 0.03	7.29 ± 1.29	19.54 ± 3.90
Christie	0.94 ± 0.85	ND	1.28 ± 0.12	0.69 ± 0.03	0.61 ± 0.37	0.15 ± 0.04	7.86 ± 3.31	0.41 ± 0.14	ND	8.12 ± 1.21	20.05 ± 3.94
CRS 15	0.40 ± 0.31	ND	1.38 ± 0.29	0.59 ± 0.30	0.81 ± 0.34	0.16 ± 0.01	9.19 ± 0.98	0.26 ± 0.01	ND	9.05 ± 2.69	21.85 ± 2.90
CRS 18	0.52 ± 0.30	ND	1.68 ± 0.14	0.57 ± 0.19	0.72 ± 0.21	0.14 ± 0.01	6.89 ± 0.59	0.38 ± 0.03	ND	6.46 ± 1.61	17.35 ± 1.44
CRS 30	0.71 ± 0.40	ND	1.29 ± 0.56	0.64 ± 0.13	0.64 ± 0.18	0.13 ± 0.02	6.07 ± 1.31	0.28 ± 0.08	0.05 ± 0.05	5.77 ± 0.81	15.58 ± 2.58
CRS 34	0.60 ± 0.45	ND	1.90 ± 0.66	0.73 ± 0.41	1.18 ± 0.41	0.18 ± 0.03	8.55 ± 2.43	0.53 ± 0.17	0.05 ± 0.08	8.30 ± 2.15	22.01 ± 3.09
CRS 35	0.69 ± 0.53	ND	1.27 ± 0.16	0.55 ± 0.13	0.55 ± 0.09	0.14 ± 0.01	7.89 ± 2.33	0.39 ± 0.26	ND	5.74 ± 2.38	17.21 ± 4.27
CRS 36	0.42 ± 0.49	0.55 ± 0.96	1.47 ± 0.16	0.54 ± 0.11	0.68 ± 0.29	0.19 ± 0.04	10.58 ± 0.93	0.31 ± 0.09	ND	6.81 ± 4.15	21.56 ± 4.52
Dolina	0.68 ± 0.84	ND	1.33 ± 0.47	0.49 ± 0.19	0.74 ± 0.38	0.15 ± 0.02	6.64 ± 1.37	0.39 ± 0.13	ND	7.28 ± 1.02	17.69 ± 2.62
Endure	0.75 ± 0.56	ND	1.86 ± 0.71	0.89 ± 0.67	1.00 ± 0.25	0.19 ± 0.05	9.10 ± 2.78	0.32 ± 0.05	0.05 ± 0.09	8.85 ± 1.26	23.01 ± 3.03
Kvarta	0.42 ± 0.24	ND	1.68 ± 0.18	0.61 ± 0.17	0.91 ± 0.25	0.15 ± 0.02	6.94 ± 2.07	0.36 ± 0.04	0.04 ± 0.06	10.67 ± 1.57	21.76 ± 3.65
Radegast	0.71 ± 0.68	ND	1.69 ± 0.75	0.58 ± 0.31	0.75 ± 0.19	0.17 ± 0.04	7.77 ± 0.80	0.38 ± 0.10	0.07 ± 0.12	7.53 ± 1.82	19.64 ± 0.61
Vesna	0.43 ± 0.31	ND	2.03 ± 0.48	0.75 ± 0.36	1.14 ± 0.26	0.20 ± 0.03	11.21 ± 0.41	0.56 ± 0.03	ND	11.42 ± 3.06	27.75 ± 3.22
average	0.60	0.04	1.57	0.63	0.81	0.16	8.22	0.38	0.02	7.94	20.39
						LF					
Amos	ND	0.52 ± 0.10	0.53 ± 0.08	0.11 ± 0.10	0.84 ± 0.24	0.19 ± 0.06	8.70 ± 2.27	0.65 ± 0.16	0.14 ± 0.13	15.40 ± 0.66	27.07 ± 3.05
Christie	0.01 ± 0.01	0.45 ± 0.05	0.07 ± 0.12	0.32 ± 0.10	0.61 ± 0.22	0.22 ± 0.03	11.81 ± 1.42	0.85 ± 0.17	0.12 ± 0.03	8.69 ± 0.66	23.16 ± 4.67
CRS 15	0.21 ± 0.04	0.34 ± 0.08	1.07 ± 0.63	0.22 ± 0.06	0.67 ± 0.23	0.18 ± 0.02	13.32 ± 1.35	0.41 ± 0.14	0.11 ± 0.01	16.05 ± 2.28	32.59 ± 2.57
CRS 18	0.11 ± 0.05	0.57 ± 0.06	0.39 ± 0.10	ND	0.65 ± 0.09	0.18 ± 0.05	8.02 ± 2.19	0.33 ± 0.07	0.03 ± 0.03	10.01 ± 3.82	20.29 ± 5.11
CRS 30	0.12 ± 0.09	0.58 ± 0.05	0.54 ± 0.24	ND	0.89 ± 0.19	0.21 ± 0.04	10.37 ± 1.52	0.42 ± 0.10	0.12 ± 0.02	11.35 ± 1.56	24.60 ± 0.78
CRS 34	0.19 ± 0.06	0.57 ± 0.14	0.39 ± 0.19	0.10 ± 0.09	0.77 ± 0.10	0.21 ± 0.03	10.79 ± 1.70	0.65 ± 0.24	0.11 ± 0.03	7.59 ± 0.96	21.36 ± 1.50
CRS 35	0.18 ± 0.08	0.06 ± 0.10	0.51 ± 0.15	0.33 ± 0.03	0.72 ± 0.29	0.20 ± 0.02	12.49 ± 1.03	0.42 ± 0.11	0.18 ± 0.05	17.94 ± 0.83	32.93 ± 0.41
CRS 36	0.08 ± 0.02	0.57 ± 0.21	0.52 ± 0.08	0.07 ± 0.06	0.74 ± 0.14	0.21 ± 0.04	12.72 ± 3.91	0.41 ± 0.07	0.11 ± 0.06	11.63 ± 1.17	27.16 ± 5.21
Dolina	0.03 ± 0.05	0.58 ± 0.08	0.59 ± 0.27	0.15 ± 0.05	0.78 ± 0.13	0.22 ± 0.04	11.49 ± 4.42	0.64 ± 0.23	0.15 ± 0.03	12.21 ± 3.51	26.84 ± 1.62
Endure	0.07 ± 0.07	0.76 ± 0.30	0.64 ± 0.19	ND	1.21 ± 0.42	0.22 ± 0.04	10.75 ± 2.19	0.35 ± 0.10	ND	15.72 ± 3.24	29.72 ± 5.12
Kvarta	ND	0.63 ± 0.19	0.45 ± 0.20	ND	0.80 ± 0.18	0.22 ± 0.05	11.57 ± 0.89	0.48 ± 0.06	0.10 ± 0.01	11.53 ± 1.99	25.78 ± 3.16
Radegast	0.02 ± 0.01	0.63 ± 0.24	0.43 ± 0.15	ND	1.04 ± 0.29	0.25 ± 0.02	10.12 ± 1.44	0.51 ± 0.09	0.11 ± 0.02	13.05 ± 3.21	26.17 ± 3.02
Vesna	0.05 ± 0.06	0.59 ± 0.33	0.35 ± 0.15	ND	0.91 ± 0.41	0.22 ± 0.04	12.03 ± 0.67	0.54 ± 0.22	0.12 ± 0.01	11.43 ± 3.94	26.26 ± 4.91
average	0.08	0.53	0.50	0.10	0.82	0.21	11.09	0.51	0.11	12.51	27.78

vones in the red clover samples tested in this study were aglycones as the hydrolysis did not have a significant effect on the profile.

This contradicts what has been reported in the vast majority of studies, which showed that glycosides and malonylglycosides were the intrinsic forms of isoflavones in red clover. Studies have found that although isoflavone glycosides are relatively stable, they are prone to hydrolysis by the enzyme β-glucosidase, which can be activated when tissues are damaged. On the other hand, malonylglycosides are found to be readily degraded to glycosides under ambient conditions (*11*). Swinny and Ryan (*10*) found that a thawing and slow-drying process after freezing the sample (vacuum drying) resulted in nearly complete profile of isoflavone aglycones, but freeze drying retained the native forms (glycosides) (*10*). Our sample processing was similar to the freeze drying reported by these scientists; however, surprisingly, the profiles of our samples were similar to the vacuum-dried samples in their study. The only difference may reside with the prefreeze-drying process, i.e., storage of samples at −5 °C after harvesting. The native isoflavones were found to be glycosides and malonylglycosides with air-dried samples harvested in other years (data not shown); therefore, the most influential factors may be the sample-processing conditions. Hydrolysis might have happened even though proper procedures and cautions were taken in keeping freshly harvested samples at −5 °C before freeze drying. Intentional hydrolysis with mineral acid certainly lead to total hydrolysis of isoflavone glycosides and their malonylglycosides; however, processing conditions such as ours, or any conditions that allow β-glucosidase to be active, e.g., damage to fresh tissue and existence of ambient water during extraction, might lead to similar results (**Figure 3**). The isoflavone profile of a red clover leaf extract was similar to that of a commercial product (**Figure 3**). The aglycones extracted after such a process may be considered "natural" or "native" since no chemical hydrolysis was performed, and that may be how commercially isoflavones are extracted from red clover although manufacturers do not include this in their proprietary technologies (*25*).

Isoflavone Concentrations in Different Parts. The average total isoflavone content of all parts excluding the flower was 17.21 mg/g DM. The total isoflavone concentrations in the leaves (including both EB and LF leaves) were the highest, with an average of 23.43 mg/g DM, followed by an average of 14.71 mg/g DM in all stems, and 13.50 mg/g DM in the petioles. The average total isoflavone content in the flower was 2.38 mg/g DM. These are higher than concentrations reported by others in respective plant organs (5, *24*, *26*, *27*). Isoflavones in the flower have been reported with inconsistent concentrations (*24*, *26*, *27*). However, as Sivesind and Seguin (*24*) recently showed, the concentration truly depended on the inflorescent stages; when inflorescence was barely palpable on the main stem, the isoflavone content was as high as in the leaf, but when the inflorescence on the main stem was discernible, the concentration was close to that in the stem. They also found that once the flower started to bloom, the isoflavone content became steadily low. The average concentration reported by Sivesind and Seguin (*24*) was 3.3 mg/g DM, which included the green buds, but in true flowers (fully opened), it was lower (ca. <1 mg/g DM). Wu et al. (*27*) studied isoflavones in the flowers of two red clovers collected from the field (cultivar unknown) and found that the concentrations were 3−6 mg/g DM, higher than the average concentration found in flowers studied in the present study. While some studies only focused on formononetin and biochanin A (*24*, *26*), we intended to study the whole spectrum of isoflavone profiles. Different conclusions may be drawn when the focus is only on the major isoflavones, as was found by Wu et al. (*27*) (see discussion below on individual compounds). Wu et al. (*27*) studied the isoflavone contents in red clovers grown in four different locations. The average total isoflavone concentration in the leaves of the four red clovers (cultivar unknown) was 19.50 mg/g DM, followed by 12.10 mg/g DM in the stems. These concentrations were both lower than what

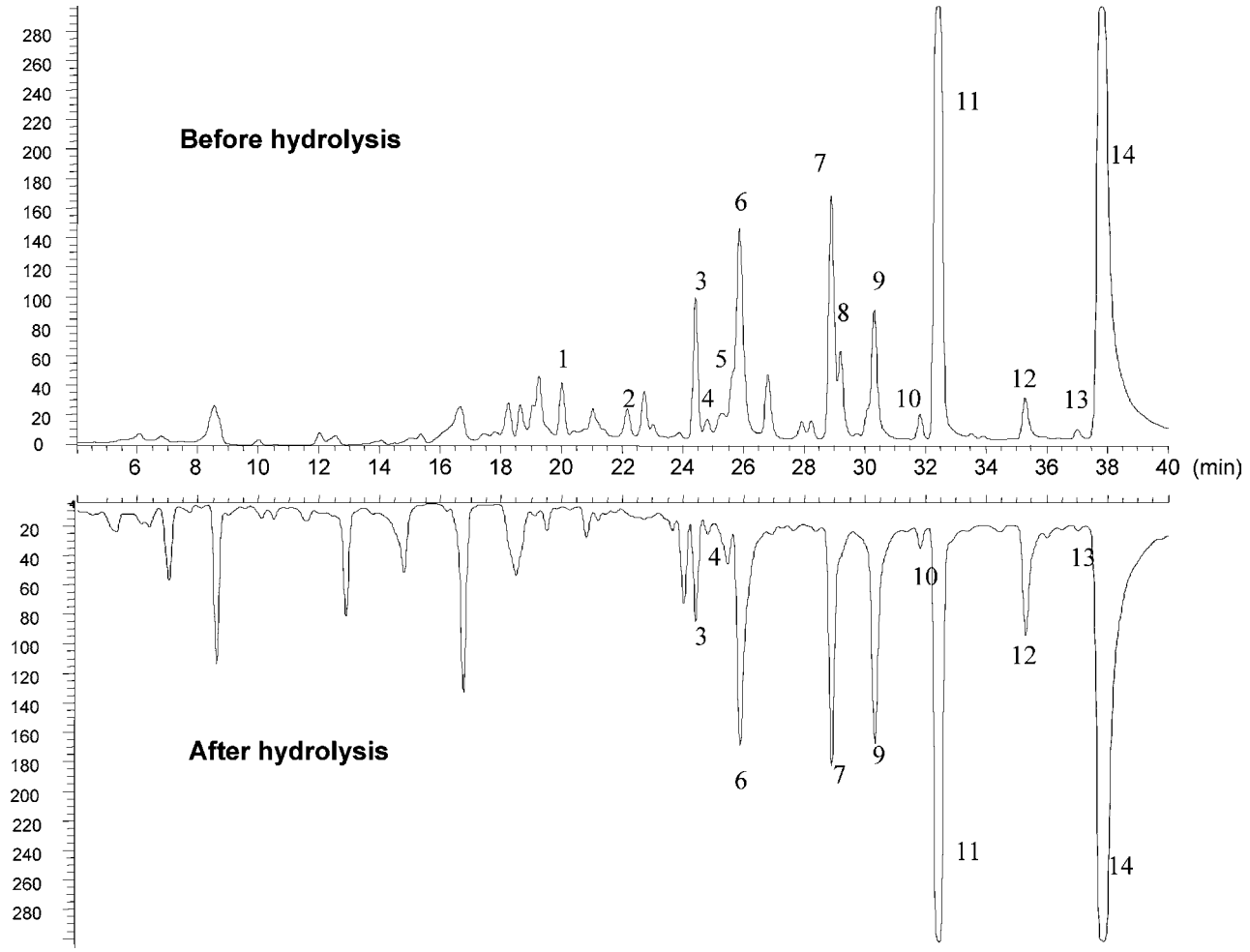

Figure 3. Isoflavone profiles before and after hydrolysis (Dolina late blooming). Peak 1, genistein-7-glucoside-6″-malonate; 2, orobol-7-glucoside-6″-malonate; 3, daidzein; 4, orobol; 5, formononetin-7-glucoside-6″-malonate; 6, glycitein; 7, genistein; 8, biochanin A-7-glucoside-6″-malonate; 9, pratensein; 10, pseudobaptigenin; 11, formononetin; 12, prunetin; 13, irilone; and 14, biochanin A.

were found in our study. Our results were also higher than the total isoflavone concentrations (8.92−12.75 mg/g DM) in 10 red clover cultivars (whole plants) reported by Sivesind and Seguin (*24*).

In terms of individual isoflavones, formononetin and biochanin A predominated the profile of all cultivars studied. The average concentrations for formononetin and biochanin A were nearly the same at 9.65 and 10.22 mg/g DM in the leaves (including samples harvested at both growth stages, EB and LF), followed by glycitein, pratensein, prunetin, genistein, daidzein, orobol, pseudobaptigenin, and irilone at 1.03, 0.82, 0.45, 0.37, 0.34, 0.28, 0.19, and 0.06 mg/g DM, respectively (**Table 2**). In the stems (both EB and LF), only formononetin was found to dominate the profile at 9.57 mg/g DM, followed by biochanin A, prunetin, glycitein, pseudobaptigenin, pratensein, genistein, daidzein, irilone, and orobol at 2.37, 0.87, 0.57, 0.40, 0.39, 0.29, 0.24, 0.01, and 0.01 mg/g DW, respectively (**Table 3**). The isoflavone profiles in the petioles were similar to those in the stems, with formononetin at 8.05 mg/g DM, followed by biochanin A, prunetin, glycitein, daidzein, genistein, pratensein, pseudobaptigenin, orobol, and irilone at 2.36, 1.09, 0.51, 0.38, 0.38, 0.36, 0.32, 0.02, and 0.02 mg/g DW, respectively (**Table 4**). While in most studies, only formononetin and biochanin A are quantified, Wu et al. developed a good method for separation and quantification of 10 isoflavone aglycones of red clover (*27*). The identities of the 10 isoflavones found in their study were nearly the same as what we found in the 13 cultivars in our study except for calycosin. We found orobol in red clovers instead of calycosin (**Table 2b**). Also interesting is the concentration of the individual isoflavones; in the study of Wu et al. (*27*), glycitein had the highest concentration in the leaf at 5.96 mg/g DM, followed by irilone, prunetin, genistein, pseudobaptigenin, bioahanin A, calycosin, formononetin, pratensein, and daidzein at 4.58, 2.14, 1.66, 1.28, 1.01, 0.96, 0.82, 0.60, and 0.51 mg/g DM, respectively (*27*). The profiles of individual isoflavones in the stems were also different from the present study; again, with glycitein on top of the list at 4.58 mg/g DM, followed by prunetin, genistein, irilone, pseudobaptigenin, daidzein, calycosin, bioahanin A, formononetin, and pratensein at 1.85, 1.37, 0.91, 0.85, 0.68, 0.67, 0.60, 0.46, and 0.15 mg/g DM, respectively (*27*). Although Wu et al. (*27*) only examined the isoflavones in the flowers of two different red clover samples, the isoflavone compositions in their study were again similar to what was found in their leaf and stem samples and significantly different from what was found in ours and other studies, which contained mainly formononetin and biochanin A as the leading isoflavones in all parts. Plant parts clearly play an important role in total and individual isoflavone concentrations. Some commercial products extracted red clover isoflavones from the flowers (*24*); however, as our results indicated, the flower would be the last plant organ one uses to extract isoflavones (**Table 5**).

Effect of Growth Stage. The impact of growth stage on the total isoflavone contents depended largely on different parts of

Table 3. Isoflavone Concentrations (mg/g Dry Weight) in Stems of the EB and LF

	daidzein	orobol	glycitein	genistein	pratensein	pseudobaptigenin	formononetin	prunetin	irilone	biochanin A	grand total
						EB					
Amos	0.07 ± 0.06	ND	0.48 ± 0.24	0.27 ± 0.17	0.42 ± 0.14	0.34 ± 0.21	8.87 ± 2.61	0.91 ± 0.37	0.02 ± 0.03	2.81 ± 0.04	14.19 ± 3.73
Christie	0.05 ± 0.04	ND	0.32 ± 0.17	0.31 ± 0.22	0.43 ± 0.39	0.26 ± 0.06	6.38 ± 1.95	0.67 ± 0.21	ND	2.80 ± 0.65	11.22 ± 3.52
CRS 15	0.03 ± 0.06	ND	0.36 ± 0.08	0.13 ± 0.05	0.43 ± 0.14	0.39 ± 0.10	10.32 ± 3.63	0.50 ± 0.11	ND	2.66 ± 0.79	14.84 ± 2.79
CRS 18	0.06 ± 0.06	ND	0.92 ± 0.31	0.10 ± 0.10	0.51 ± 0.14	0.67 ± 0.09	17.51 ± 2.24	1.06 ± 0.25	0.03 ± 0.05	3.08 ± 0.96	23.94 ± 2.62
CRS 30	0.46 ± 0.52	ND	0.68 ± 0.58	0.34 ± 0.22	0.35 ± 0.20	0.41 ± 0.11	10.53 ± 3.50	0.97 ± 0.22	ND	2.33 ± 1.74	16.07 ± 3.88
CRS 34	ND	ND	0.53 ± 0.25	0.17 ± 0.03	0.19 ± 0.08	0.39 ± 0.15	8.78 ± 0.84	0.86 ± 0.39	0.03 ± 0.06	1.39 ± 0.34	12.34 ± 1.78
CRS 35	0.08 ± 0.07	ND	0.43 ± 0.13	0.27 ± 0.04	0.35 ± 0.17	0.37 ± 0.14	10.02 ± 1.01	0.79 ± 0.11	0.03 ± 0.03	2.30 ± 1.36	14.63 ± 1.06
CRS 36	0.29 ± 0.21	ND	1.20 ± 0.73	0.36 ± 0.10	0.78 ± 0.35	0.53 ± 0.24	15.49 ± 1.46	1.02 ± 0.07	ND	3.79 ± 0.95	23.46 ± 2.98
Dolina	0.09 ± 0.09	ND	0.63 ± 0.21	0.53 ± 0.42	0.45 ± 0.26	0.45 ± 0.17	12.22 ± 1.36	1.22 ± 0.41	ND	2.68 ± 1.90	18.27 ± 3.52
Endure	0.04 ± 0.06	ND	0.82 ± 0.09	0.35 ± 0.29	0.47 ± 0.34	0.54 ± 0.10	15.75 ± 0.99	1.00 ± 0.38	ND	2.78 ± 1.55	21.74 ± 2.39
Kvarta	0.14 ± 0.15	ND	0.85 ± 0.23	0.47 ± 0.11	0.67 ± 0.31	0.55 ± 0.20	13.21 ± 1.34	1.26 ± 0.23	ND	3.95 ± 0.58	21.10 ± 2.69
Radegast	0.41 ± 0.71	ND	1.00 ± 0.63	0.30 ± 0.31	0.29 ± 0.10	0.50 ± 0.09	11.96 ± 0.82	0.99 ± 0.18	ND	1.34 ± 0.93	16.79 ± 3.46
Vesna	0.08 ± 0.07	ND	0.72 ± 0.23	0.36 ± 0.24	0.43 ± 0.19	0.51 ± 0.13	11.14 ± 1.52	1.15 ± 0.45	ND	2.46 ± 1.84	16.85 ± 3.59
average	0.14	0	0.69	0.31	0.44	0.46	11.71	0.95	0.01	2.64	17.34
						LF					
Amos	0.26 ± 0.13	ND	0.49 ± 0.21	0.28 ± 0.09	0.31 ± 0.11	0.40 ± 0.14	7.32 ± 2.49	0.93 ± 0.15	0.03 ± 0.02	1.49 ± 0.66	11.49 ± 3.83
Christie	0.47 ± 0.06	ND	0.39 ± 0.06	0.32 ± 0.20	0.32 ± 0.15	0.27 ± 0.01	6.13 ± 0.99	0.69 ± 0.06	ND	2.39 ± 1.52	10.97 ± 2.13
CRS 15	0.25 ± 0.43	ND	0.35 ± 0.08	0.13 ± 0.05	0.21 ± 0.06	0.31 ± 0.05	7.65 ± 0.64	0.46 ± 0.21	ND	0.94 ± 0.51	10.30 ± 1.28
CRS 18	0.55 ± 0.55	ND	0.32 ± 0.03	0.13 ± 0.18	0.20 ± 0.13	0.27 ± 0.02	6.43 ± 1.74	0.67 ± 0.26	0.04 ± 0.04	0.88 ± 0.89	9.49 ± 2.60
CRS 30	0.51 ± 0.39	ND	0.46 ± 0.08	0.42 ± 0.19	0.61 ± 0.33	0.37 ± 0.05	7.02 ± 1.66	0.71 ± 0.11	0.03 ± 0.03	2.66 ± 1.82	12.79 ± 3.16
CRS 34	0.10 ± 0.17	0.04 ± 0.08	0.44 ± 0.09	0.13 ± 0.07	0.22 ± 0.09	0.27 ± 0.03	4.96 ± 0.30	0.62 ± 0.11	ND	0.91 ± 0.53	7.68 ± 0.66
CRS 35	0.29 ± 0.29	0.06 ± 0.10	0.39 ± 0.13	0.45 ± 0.25	0.44 ± 0.10	0.30 ± 0.08	8.54 ± 2.81	1.00 ± 0.26	ND	3.45 ± 1.96	14.92 ± 3.26
CRS 36	0.62 ± 0.48	0.06 ± 0.10	0.57 ± 0.22	0.24 ± 0.05	0.24 ± 0.13	0.38 ± 0.12	8.64 ± 2.77	0.65 ± 0.25	ND	1.81 ± 0.79	13.22 ± 3.03
Dolina	0.27 ± 0.26	ND	0.42 ± 0.15	0.31 ± 0.31	0.31 ± 0.18	0.31 ± 0.02	7.18 ± 1.97	0.98 ± 0.47	0.03 ± 0.03	4.01 ± 0.02	13.82 ± 2.84
Endure	0.20 ± 0.16	ND	0.49 ± 0.23	0.23 ± 0.17	0.38 ± 0.15	0.36 ± 0.10	7.71 ± 2.50	0.50 ± 0.16	ND	2.74 ± 1.40	12.61 ± 3.32
Kvarta	0.41 ± 0.34	ND	0.48 ± 0.08	0.19 ± 0.01	0.26 ± 0.10	0.36 ± 0.05	6.17 ± 2.56	0.64 ± 0.13	0.01 ± 0.02	0.91 ± 0.30	9.43 ± 3.19
Radegast	0.05 ± 0.09	ND	0.65 ± 0.04	0.54 ± 0.23	0.53 ± 0.15	0.52 ± 0.03	12.34 ± 0.44	1.32 ± 0.19	ND	3.64 ± 1.30	19.60 ± 1.62
Vesna	0.50 ± 0.22	ND	0.46 ± 0.06	0.31 ± 0.28	0.34 ± 0.18	0.37 ± 0.08	6.45 ± 0.65	0.95 ± 0.24	ND	1.35 ± 0.94	10.74 ± 1.66
average	0.34	0.01	0.45	0.28	0.34	0.35	7.43	0.78	0.01	2.09	12.08

Table 4. Isoflavone Concentrations (mg/g Dry Weight) in Petioles of the EB and the LF

	daidzein	orobol	glycitein	genistein	pratensein	pseudobaptigenin	formononetin	prunetin	irilone	biochanin A	grand total
						EB					
Amos	0.23 ± 0.10	0.07 ± 0.12	0.53 ± 0.22	0.46 ± 0.12	0.35 ± 0.11	0.36 ± 0.13	10.01 ± 1.23	1.43 ± 0.26	ND	2.64 ± 1.13	16.07 ± 2.73
Christie	0.22 ± 0.09	ND	0.60 ± 0.31	0.34 ± 0.11	0.33 ± 0.03	0.25 ± 0.05	6.62 ± 1.29	0.89 ± 0.08	ND	2.60 ± 0.07	11.84 ± 0.91
CRS 15	0.31 ± 0.09	0.15 ± 0.27	0.51 ± 0.15	0.22 ± 0.04	0.43 ± 0.05	0.30 ± 0.03	8.49 ± 0.87	0.68 ± 0.12	ND	2.92 ± 1.05	14.00 ± 1.76
CRS 18	0.13 ± 0.03	ND	0.53 ± 0.15	0.16 ± 0.21	0.38 ± 0.13	0.29 ± 0.04	9.26 ± 1.69	1.08 ± 0.11	ND	3.01 ± 1.65	14.85 ± 3.35
CRS 30	0.61 ± 0.33	ND	0.76 ± 0.35	0.38 ± 0.07	0.27 ± 0.02	0.25 ± 0.01	6.11 ± 0.93	0.95 ± 0.10	0.06 ± 0.06	1.81 ± 0.67	11.19 ± 1.57
CRS 34	0.05 ± 0.05	0.37 ± 0.22	0.48 ± 0.08	0.23 ± 0.07	0.27 ± 0.03	0.26 ± 0.00	7.31 ± 0.19	0.99 ± 0.15	ND	2.34 ± 1.43	12.06 ± 1.58
CRS 35	0.28 ± 0.07	0.15 ± 0.25	0.51 ± 0.22	0.45 ± 0.10	0.36 ± 0.05	0.31 ± 0.12	9.83 ± 0.82	1.09 ± 0.17	0.02 ± 0.03	2.80 ± 0.40	15.80 ± 1.25
CRS 36	0.37 ± 0.11	ND	0.53 ± 0.07	0.37 ± 0.14	0.39 ± 0.11	0.35 ± 0.03	12.63 ± 1.08	1.09 ± 0.10	0.28 ± 0.44	3.41 ± 1.48	19.41 ± 2.50
Dolina	0.22 ± 0.04	ND	0.48 ± 0.01	0.34 ± 0.11	0.27 ± 0.06	0.28 ± 0.05	7.34 ± 3.19	1.06 ± 0.26	ND	1.55 ± 0.65	11.53 ± 3.95
Endure	0.60 ± 0.48	ND	0.46 ± 0.19	0.44 ± 0.53	0.32 ± 0.18	0.32 ± 0.12	10.99 ± 1.22	0.85 ± 0.29	0.03 ±	3.15 ± 3.56	17.18 ± 4.30
Kvarta	0.18 ± 0.04	ND	0.53 ± 0.08	0.42 ± 0.05	0.40 ± 0.13	0.33 ± 0.02	9.30 ± 2.23	1.23 ± 0.22	0.02 ± 0.03	2.68 ± 0.61	15.07 ± 2.44
Radegast	0.16 ± 0.09	0.11 ± 0.18	0.57 ± 0.19	0.36 ± 0.03	0.33 ± 0.03	0.33 ± 0.02	9.41 ± 1.62	1.19 ± 0.04	0.02 ± 0.03	3.15 ± 0.64	15.61 ± 2.39
Vesna	0.21 ± 0.07	ND	0.84 ± 0.34	0.51 ± 0.19	0.54 ± 0.24	0.37 ± 0.02	8.63 ± 5.64	1.39 ± 0.23	ND	3.88 ± 0.86	16.36 ± 3.91
average	0.27	0.05	0.56	0.36	0.36	0.31	8.92	1.07	0.03	2.77	14.69
						LF					
Amos	0.43 ± 0.22	ND	0.41 ± 0.09	0.38 ± 0.08	0.30 ± 0.04	0.29 ± 0.05	6.92 ± 0.94	1.28 ± 0.15	ND	1.69 ± 0.42	11.69 ± 1.76
Christie	0.32 ± 0.28	ND	0.44 ± 0.14	0.35 ± 0.05	0.33 ± 0.06	0.28 ± 0.03	7.14 ± 1.20	1.04 ± 0.08	0.03 ± 0.03	2.26 ± 0.74	12.19 ± 1.89
CRS 15	0.60 ± 0.27	ND	0.37 ± 0.03	0.38 ± 0.06	0.31 ± 0.03	0.20 ± 0.09	6.05 ± 1.63	0.89 ± 0.02	ND	2.07 ± 0.38	10.86 ± 2.14
CRS 18	0.46 ± 0.04	ND	0.39 ± 0.09	0.20 ± 0.29	0.31 ± 0.04	0.32 ± 0.09	7.77 ± 1.23	1.10 ± 0.24	ND	1.65 ± 0.53	12.22 ± 2.27
CRS 30	0.66 ± 0.32	ND	0.56 ± 0.15	0.60 ± 0.41	0.44 ± 0.07	0.40 ± 0.12	7.43 ± 2.11	1.20 ± 0.13	0.04 ± 0.03	1.65 ± 0.22	12.98 ± 1.44
CRS 34	0.23 ± 0.05	ND	0.45 ± 0.04	0.23 ± 0.08	0.30 ± 0.08	0.27 ± 0.01	5.08 ± 0.35	0.87 ± 0.12	ND	1.61 ± 0.53	9.04 ± 0.98
CRS 35	0.82 ± 0.53	ND	0.45 ± 0.04	0.45 ± 0.24	0.35 ± 0.05	0.29 ± 0.07	7.39 ± 1.29	1.20 ± 0.32	ND	2.48 ± 0.40	13.43 ± 1.70
CRS 36	0.51 ± 0.11	ND	0.55 ± 0.15	0.52 ± 0.28	0.48 ± 0.15	0.40 ± 0.14	8.97 ± 0.85	1.28 ± 0.57	0.02 ± 0.03	2.84 ± 0.06	15.57 ± 2.01
Dolina	0.66 ± 0.08	ND	0.42 ± 0.07	0.42 ± 0.08	0.31 ± 0.07	0.30 ± 0.05	5.76 ± 0.47	1.18 ± 0.08	ND	1.53 ± 0.65	10.58 ± 0.40
Endure	0.30 ± 0.16	ND	0.50 ± 0.40	0.75 ± 0.38	0.61 ± 0.38	0.38 ± 0.15	8.48 ± 1.13	1.14 ± 0.46	ND	3.20 ± 0.64	15.35 ± 2.36
Kvarta	0.48 ± 0.09	ND	0.49 ± 0.17	0.27 ± 0.15	0.31 ± 0.10	0.31 ± 0.01	7.54 ± 1.26	0.86 ± 0.20	0.03 ± 0.04	1.81 ± 0.87	12.08 ± 2.38
Radegast	0.50 ± 0.11	ND	0.56 ± 0.10	0.21 ± 0.14	0.20 ± 0.07	0.48 ± 0.05	8.13 ± 0.80	1.08 ± 0.11	0.02 ± 0.03	0.94 ± 0.43	12.12 ± 0.44
Vesna	0.41 ± 0.23	ND	0.47 ± 0.16	0.46 ± 0.18	0.38 ± 0.11	0.33 ± 0.06	6.73 ± 1.42	1.25 ± 0.21	ND	1.76 ± 0.69	11.79 ± 2.69
average	0.49	0.00	0.47	0.40	0.36	0.33	7.18	1.11	0.01	1.96	12.30

the red clover plant. In the leaves, the average total isoflavone content significantly increased by 30% from 20.39 mg/g DM in the EB stage to 26.46 mg/g DM in the LF stage, whereas in the stems and petioles, the total isoflavone content decreased significantly as the plants grew toward maturity. In the stems, the average concentration decreased from 17.34 mg/g DM in the EB to 12.08 mg/g DM in the LF stage, while in the petioles, it decreased from 14.69 to 12.30 mg/g DM.

In terms of individual isoflavones, both of the dominant isoflavones formanonetin and biochanin A increased significantly in LF leaves than in EB leaves. The concentration of formononetin increased from 8.22 to 11.09 mg/g DM, while biochanin A increased from 7.94 mg/g DM in the EB leaves to 12.51 mg/g DM in the LF leaves (**Table 2**). These two compounds together were on average 79 and 85% of the total isoflavone content in EB and LF leaf samples, respectively

Table 5. Isoflavone Concentrations (mg/g dry weight) in Flowers of the LF

	genistein	pratensein	pseudobaptigenin	formononetin	prunetin	biochanin A	grand total
Amos	0.12 ± 0.06	0.42 ± 0.11	0.14 ± 0.03	1.06 ± 0.54	0.28 ± 0.09	1.06 ± 0.23	3.14 ± 0.91
Christie	0.09 ± 0.11	0.13 ± 0.22	0.12 ± 0.02	0.6 ± 0.21	0.31 ± 0.15	0.69 ± 0.26	1.94 ± 0.51
CRS 15	0.02 ± 0.04	0.19 ± 0.17	0.14 ± 0.02	1.12 ± 0.64	0.18 ± 0.02	0.71 ± 0.21	2.37 ± 0.58
CRS 18	0.05 ± 0.05	0.15 ± 0.14	0.12 ± 0.01	0.76 ± 0.31	0.19 ± 0.04	0.79 ± 0.41	2.07 ± 0.69
CRS 30	0.10 ± 0.04	0.17 ± 0.16	0.12 ± 0.01	0.57 ± 0.26	0.29 ± 0.02	0.61 ± 0.07	1.86 ± 0.50
CRS 34	0.08 ± 0.02	0.27 ± 0.23	0.16 ± 0.03	0.69 ± 0.29	0.19 ± 0.05	0.95 ± 0.39	2.33 ± 0.94
CRS 35	0.11 ± 0.07	0.21 ± 0.22	0.12 ± 0.02	0.83 ± 0.20	0.19 ± 0.08	0.90 ± 0.43	2.46 ± 0.79
CRS 36	0.11 ± 0.07	0.29 ± 0.28	0.16 ± 0.05	0.88 ± 0.19	0.30 ± 0.22	0.98 ± 0.34	2.73 ± 0.67
Dolina	0.11 ± 0.06	0.38 ± 0.35	0.13 ± 0.03	0.84 ± 0.36	0.22 ± 0.06	1.17 ± 0.24	2.85 ± 0.96
Endure	0.09 ± 0.03	0.49 ± 0.13	0.16 ± 0.01	1.03 ± 0.60	0.22 ± 0.06	1.24 ± 0.21	3.23 ± 0.89
Kvarta	ND	0.10 ± 0.17	0.11 ± 0.01	0.47 ± 0.25	0.28 ± 0.16	0.66 ± 0.14	1.68 ± 0.80
Radegast	0.02 ± 0.04	0.16 ± 0.14	0.13 ± 0.02	0.90 ± 0.26	0.19 ± 0.02	0.80 ± 0.30	2.24 ± 0.27
Vesna	0.05 ± 0.04	0.16 ± 0.13	0.18 ± 0.10	0.61 ± 0.20	0.36 ± 0.28	0.70 ± 0.32	2.05 ± 0.74
average	0.07	0.24	0.14	0.80	0.25	0.87	2.38

Table 6. Total Isoflavone Content in Different Parts of Diploid and Tetrapoid Red Clovers (mg/g DM)

	chromosome number	LE[a]	LL	SE	SL	PE	PL	average
Christie	d[b]	20.05	23.16	11.22	10.97	11.84	12.19	14.91
CRS 15	d	21.85	32.59	14.84	10.30	14.00	10.86	17.41
CRS 30	d	15.58	24.60	16.07	12.79	11.19	12.98	15.53
CRS 34	d	22.01	21.36	12.34	7.68	12.06	9.04	14.08
CRS 35	d	17.21	32.93	14.63	14.92	15.80	13.43	18.15
CRS 36	d	21.56	27.16	23.46	13.22	19.41	15.57	20.06
Endure	d	23.01	29.72	21.74	12.61	17.18	15.35	19.93
Amos	t	19.54	27.07	14.19	11.49	16.07	11.69	16.67
CRS 18	t	17.35	20.29	23.94	9.49	14.85	12.22	16.36
Dolina	t	17.69	26.84	18.27	13.82	11.53	10.58	16.46
Kvarta	t	21.76	25.78	21.10	9.43	15.07	12.08	17.54
Radegast	t	19.64	26.17	16.79	19.60	15.61	12.12	18.32
Vesna	t	27.75	26.26	16.85	10.74	16.36	11.79	18.29
average d		20.18 ± 2.77	27.36 ± 4.57	16.33 ± 4.61	11.78 ± 2.35	14.50 ± 3.09	12.77 ± 2.34	17.15 ± 2.39
average t		20.62 ± 3.84	25.40 ± 2.55	18.53 ± 3.48	12.43 ± 3.87	14.92 ± 1.76	11.75 ± 0.61	17.27 ± 0.90

[a] LE, leaf in EB; LL, leaf in LF; SE, stem in EB; SL, stem in LF; PE, petiole in EB; and PL, petiole in LF. [b] d, diploid; t, tetraploid.

(**Table 2**). In the stem, both of these two isoflavones decreased in LF samples; however, formononetin dropped significantly from 11.71 to 7.43 mg/g DM, whereas biochanin A was at lower concentrations and decreased only slightly from 2.64 mg/g DM in the EB stems to 2.09 mg/g DM in the LF stems (**Table 3**). These two compounds were 83 and 79% of the total isoflavone content; percentagewise, similar to what was found in the leaves, however, formononetin alone had 68 and 62% of the total isoflavones in the EB and LF stems, respectively. The changes of the major isoflavones in the petioles were similar to those in the stems, i.e., decreased concentration in LF samples. Formanonetin was found to be at 8.92 mg/g DM in the EB petioles and 7.18 mg/g DM in the LF petioles, while biochanin A decreased from 2.77 to 1.96 mg/g DM. These two compounds again had 80 and 74% of the total isoflavones in the petioles in EB and LF petioles, in which 61 and 58% were from formononetin, respectively (**Table 4**). Other minor isoflavones identified in this study also varied significantly as the red clover plants grow toward maturity; however, their impact on the total isoflavone concentrations is not considered as significant as formononetin and biochanin A (**Tables 2**−**4**). Flowers also contained mainly formononetin but at a significantly lower concentration (**Table 5**).

Cultivar Effect on Isoflavone Concentrations. The average total isoflavone contents of the 13 cultivars (including EB and LF samples; excluding flowers) varied significantly by 35% from 14.91 mg/m DM in the cultivar Christie to 20.06 mg/m DM in our new breeding selection line CRS 36. Among the 13 cultivars studied, seven of them, Christie, CRS 15, CRS 30, CRS 34, CRS 35, CRS 36, and Endure, were diploid cultivars and six others, Amos, CRS 18, Dolina, Kvarta, Radegast, and Vesna, were tetraploid cultivars. There were no significant differences found between the diploids and the tetraploids in total isoflavone contents (**Table 6**). The fact that different cultivars had different isoflavone levels is suggestive of the genetic impact on the biosynthesis of isoflavones; thus, isoflavone concentrations can be potentially elevated to higher levels through breeding, which in turn favors the extraction and processing of isoflavones for the development of nutraceuticals and food supplements.

Most quantitative studies on isoflavone concentrations are focused on formononetin and biochanin A. Although these two isoflavones are the most predominant in the red clovers, profiles and concentrations of other isoflavones are important for better understanding the mechanisms of biosynthesis, hence leading to targeted breeding of isoflavone rich new cultivars and better quality of nutraceuticals and functional foods based on red clover. In addition, as discussed above, the composition of isoflavones in different red clovers may be significantly different; the major isoflavones of certain red clovers may not be formononetin and biochanin A as was found by Wu et al. (*27*).

Despite the observations that concentrations of nearly all major and minor isoflavones varied significantly among the 13 cultivars examined in this study, there were no significant differences found in the chemical compositions. All cultivars had formononetin and biochanin A as the predominating isoflavones, in all leaf, stem, petiole, and flower samples (**Tables**

2−**5**). Considering that all plants were grown in the greenhouse under the same environmental conditions, the differences between different cultivars, whether in total or individual isoflavones, are highly likely to be caused by genetics. Genetic variation has been found to play the most important role in the production of plant secondary metabolites in many crops including the clovers (*24*). This gives strong justification for breeding programs targeting specific phytochemicals such as isoflavones in red clover.

Conclusions. Red clover is a rich source of isoflavones. However, the native forms of isoflavones, i.e., the glycosides and the malonylglycosides, can be affected by several factors, particularly how samples are harvested and stored before analysis. Given the fact that most literature supports aglycones being the biologically active form, the total isoflavone content and the aglycone profile (different isoflavones) may be of more importance. The present study showed that among the 13 red clover cultivars studied, the isoflavone compositions were similar; however, the total and individual concentrations differed significantly. A marked difference was also found with the isoflavone concentration and composition in different plant parts. Leaves of the red clover plant had the highest concentration among all plant parts, and leaves harvested at LF showed even higher total isoflavone concentrations than those harvested before blooming. A higher concentration means less solvent used in extraction. Also, the harvesting time appears to be as important; in all parts of the red clover plant, the isoflavone content was higher after flowering. Findings in this study therefore provide important information for further studies on the utilization of red clover as a source for nutraceuticals and functional foods.

ACKNOWLEDGMENT

We thank Matt Mckague for his technical contribution.

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Received for review May 23, 2006. Accepted June 20, 2006. This project was fully funded by Agriculture & Agri-Food Canada A-Base Funds. This paper is Scientific Publication #S251 of the Food Research Program, Agriculture & Agri-Food Canada, Guelph, Ontario.

JF0614589