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Effects of the Environment, Cultivar, Maturity, and Preservation Method on Red Clover Isoflavone Concentration

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Red clover (*Trifolium pratense* L.) contains isoflavones that are of interest because of their benefits for human health as well as their adverse effects on the fertility of farm animals. A series of field experiments was conducted in Sainte-Anne-de-Bellevue, QC, Canada, to determine 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 multi-year, multisite trial, the total isoflavone concentration in 10 cultivars ranged between 8923 and 12 753 μg g^{-1} of DM averaged across sites, harvests, and years. Despite strong environmental effects, the cultivar "Start" consistently had the lowest isoflavone concentrations, with few differences observed among other cultivars. Across stages of maturity, leaves were found to have the highest isoflavone concentration followed by stems and inflorescences (11 970, 4896, and 3297 μg g^{-1} of DM, respectively). Changes in isoflavone concentrations with increasing maturity varied depending on the plant part. Overall, highest isoflavone concentrations were found in leaves and stems during the vegetative stages, with the formononetin concentration declining until plants initiated flowering, especially in stems, with concentrations then stabilized in both parts. Upon initiation, inflorescences contained similar isoflavone concentrations than leaves, but concentrations decreased rapidly during flower development to fall even below those observed in stems. Inflorescences then had isoflavone concentrations that were as much as 11 times lower than leaves. Fresh herbage contained higher formononetin and total isoflavone concentrations than did silage and hay (14 464, 12 200, and 11 604 μg g^{-1} of DM, respectively). The isoflavone concentration in field-grown red clover is thus high but can be affected by a range of agronomic factors.

KEYWORDS: Red clover; *Trifolium pratense* L.; isoflavone; phytoestrogens; biochanin A; formononetin; cultivars; maturity

INTRODUCTION

Studies have shown that isoflavones may lower blood cholesterol levels (*1*, *2*) and be useful in the prevention and treatment of cancer (*3*), bone loss (*4*), and symptoms associated with menopause (*5*). Red clover is a legume species that contain the isoflavones daidzein, genistein, formononetin, and biochanin A, with the latter two being found in especially high concentrations (*6*−*9*). The isoflavone concentration in red clover herbage has been reported to be 2−10 times more than in soybean seeds, the more common source of isoflavones (*10*). Products containing extracts or powered red clover material are thus currently being sold as nonprescription food supplements.

Isoflavones are also of interest because of their biological activity in farm animals. Consumption of forages with high isoflavone concentrations has been demonstrated to cause reproductive problems in sheep and cows (*11*, *12*). Alternatively, isoflavones may have some desirable effects on animals for slaughter. Finishing lambs fed red clover with high levels of the isoflavone formononetin gain weight more quickly than lambs fed low formononetin red clover or ryegrass (*13*).

Isoflavone concentration in legumes is controlled by both genetic and environmental factors. Early studies reported differences in isoflavone concentration among red clover cultivars (*6*, *14*). Dedio and Clark (*6*) reported 2.7- and 2-fold variation in formononetin and biochanin A, respectively, among 16 field-grown cultivars sampled at a single date. Environmental factors have also been reported to affect red clover isoflavone concentration. In a greenhouse experiment, formononetin concentration in expanded leaves was reported to be 28% higher in red clover plants grown in a 17/13 °C temperature regimen as opposed to 23/15 °C (*7*).

Management may also affect red clover isoflavone concentration (*7*). It was observed that when the first harvest was delayed by 21 days, the formononetin concentration decreased by 39%. For the second harvest, the plots that were allowed the shortest regrowth had the highest formononetin concentration. Differences between harvest dates might be attributable to differences

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in plant maturity at harvest. A decrease of 57% in the formononetin concentration was reported between the late vegetative and the dying inflorescence stages in greenhouse grown plants (*7*).

Isoflavones do not exist in uniform concentrations throughout the plant. It has been reported that stems and petioles contain lower concentrations of formononetin and biochanin A than leaves when sampled various times during the growing season (*6*). Wu et al. (*9*) reported that, when averaged over red clover plants collected in four different environments at one point in time, total isoflavones were greatest in leaves, intermediate in stems, and lowest in flowers. However, it has also been reported that flowers contain the highest total isoflavone concentration, followed by leaves and stems, when measured at a single sampling date (*8*). It is not known how the isoflavone concentration evolves with maturity in inflorescences, which are the most commonly used part by nutraceutical manufacturers (*15*).

Finally, preservation and storage may have an effect on the isoflavone concentration in red clover. This is especially relevant for herbage to be used as animal feed. Red clover silage has been reported to contain an 18% higher isoflavone concentration than nonensiled wilted herbage (*16*). Also, in subterranean clover (*Trifolium subterraneum* L.), it was observed that isoflavone concentrations in dried samples were up to 50% lower than in fresh or frozen material (*17*).

Despite the current importance of red clover as a source of isoflavone for the nutraceutical industry, as well as its widespread use as a feed for farm animals, there has been to date no comprehensive study looking at factors affecting isoflavone concentration in field-grown red clover. We thus designed a set of three field experiments to determine how the environment, cultivar, plant maturity, plant part, and storage method affect red clover isoflavone concentration.

MATERIALS AND METHODS

General Site Description and Management. Three different experiments were conducted in four red clover fields established in either 2002 (field A) or 2003 (fields B, C, and D) in Sainte-Anne-de-Bellevue, QC, Canada (45°25′45″ N, 73°56′00″ W). Soil types were as follows: field A, a Chicot fine sandy loam; field B, a Bearbrook clay; field C, a Chateauguay clay loam; and field D, a St. Bernard clay loam. All plots (5 × 1.35 m) were seeded in early May at a rate of 10 kg ha^{-1} using a disk drill (Fabro, Swift Current, SK, Canada). Seeds were inoculated prior to seeding with a peat-based rhizobial inoculant (Nitragin, Milwaukee, WI). Cultivars of red clover seeded varied depending on the experiment. In the seeding year, all fields received 400 kg ha^{-1} of 5−20−20 ($N-P_2O_5-K_2O$) fertilizer in May just prior to seeding and 225 kg ha^{-1} of 0−15−30 the first week of September. In post-seeding years, fields received 250 kg ha^{-1} of 0−15−30 in the spring and 250 kg ha^{-1} of 0−18−36 after the first harvest and in the first week of September. Fertilization was done according to local recommendations for red clover forage production (*18*).

Cultivar Evaluation Experiment. A total of 10 red clover cultivars recommended for forage production in Québec at the onset of experimentation (AC Charlie, Azur, Belle, Concorde, Prima, Ram, Scarlett, Start, Tempus, and Walter) were seeded on May 15, 2002 in field A and May 12, 2003 in field B. Each cultivar was replicated 4 times in a randomized complete block design. Cultivars were grown for two consecutive years, the seeding year and the first post-seeding year (2002−2003 for field A and 2003−2004 for field B) and were harvested twice in each year. A 0.6 by 4.4 m area was cut in the center of each plot at each harvest to a 7 cm stubble height using a flail forage harvester (Swift Machine and Welding, Swift Current, SK, Canada), when 10% of the plants produced flowers. Field A was harvested on August 2 and October 25, 2002 and June 20 and July 29, 2003. Field B was harvested on July 30 and August 29, 2003 and June 11 and July 21, 2004. At the time of harvest, 30 g samples of mixed, chopped fresh plant material were frozen immediately on dry ice and stored at −20 °C for subsequent isoflavone extraction in the laboratory. Representative samples of harvested herbage were also obtained from each plot, dried in a forced-air oven at 60 °C for 48 h, and weighted to determine the dry matter (DM) content.

Maturity and Plant Parts Experiment. Two cultivars, "Azur" and "Start", were seeded on May 12, 2003 in field C. Each cultivar was replicated 4 times in a randomized complete block design. Beginning in May 2004, plants were harvested by hand at specific growth stages described in Ohlsson and Weding (*19*), ranging from early vegetative through late flowering. Plant samples were collected at 8 stages as follows: stage 1 (vegetative plants with three trifoliate leaves), 2 (vegetative plants with four trifoliate leaves), 3 (vegetative plants with five trifoliate leaves), 4 (inflorescence of main stem palpable), 5 (single buds on main stem discernible), 6 (at least one open flower on inflorescence of main stem), 7 (inflorescence of main stem at least halfway past flower), and 8 (inflorescence of main stem past flower with sepals still green). At each sampling, samples were further divided into leaves, stems, and inflorescences fractions. Subsamples (30 g) from each part were finely chopped and placed on dry ice and stored at −20 °C until isoflavone extraction. At each sampling, representative samples were also dried in a forced-air oven at 60 °C for 48 h and weighted to determine the DM content.

Preservation Method Experiment. Plots of the cultivar "Concorde" were established on May 12, 2003 in field D. On July 21, 2004, at the second harvest of the post-seeding year, herbage was harvested using a flail forage harvester (Swift Machine and Welding, Swift Current, SK, Canada) as previously described and received one of three preservation treatments: (i) as hay after 2 days of field-curing to a DM content of 90%, (ii) as silage after ensiling wilted herbage (40% DM) in mini-silos for 50 days as described by Seguin and Mustafa (*20*), or (iii) none (fresh herbage). Upon treatment, 30 g subsamples were kept frozen at −20 °C until isoflavone extraction. Each treatment was replicated 4 times. Dry matter content of each sample was determined by drying representative samples in a forced-air oven at 60 °C for 48 h.

Isoflavone Extraction. Sample extraction was performed using a slightly modified version of the protocol of Petterson and Kiessling (*21*). Briefly, 1 g of plant material was ground with sand with a mortar and pestle, mixed with 2 mL of distilled water, and incubated at 37 °C for 30 min in a water bath. A total of 16 mL of ethanol and 2 mL of 3 M HCl were added, and samples were mixed and then heated to boiling. Extracts were allowed to cool, and then 2 mL of extract was removed and centrifuged at 8000 rpm for 8 min. The supernatant was loaded onto SepPak C-18 cartridges (Waters Canada, Mississauga, ON, Canada) as follows: cartridges were first equilibrated with 5 mL of methanol and washed with 5 mL of deionized water. A total of 1 mL of extract was then mixed with 3 mL of deionized water and allowed to enter the cartridge. Columns were then washed with 2 mL of 20% methanol and eluted with 2 mL of 80% methanol. Samples were then stored at −20 °C until HPLC analysis. Most isoflavones in red clover are glycosylated with only a small percentage present in the aglycone form (*22*, *23*). Red clover glucosylated isoflavones are however highly unstable. They are thermolabile and are also quickly broken down to their corresponding aglucones form by native β-glucosidases upon the disruption of cell compartmentalization during the extraction process (*23*). It was reported that the present extraction method results in the conversion of red clover isoflavones into their corresponding aglucones (*21*). Therefore, the quantification of isoflavones in this study represents the total isoflavone concentration in red clover, including glucosylated and aglucone forms.

HPLC Analyses. HPLC analyses were performed on a Waters chromatograph system (Waters, Milford, MA) consisting of two pumps (model 510), a WISP autosampler (model 712), and a UV absorbance detector (model 441). The system was equipped with a C18 reverse-phase column (Bondapak, 10 μm, 3.9 × 300 mm; Millipore, Milford, MA). A total of 100 μL of filtered extract was injected for each analysis. Separation and elution of isoflavones was achieved with the following gradient method using a flow rate of 1 mL min^{-1}. Elution of isoflavones was performed using a linear gradient system from 20% methanol and

Table 1. Analysis of Variance of the Isoflavone Concentration of Red Clover Cultivars Grown for 2 Consecutive Years at Two Sites in Sainte-Anne-de-Bellevue, QC, Canada, in 2002–2004 and Harvested Twice Per Year[a]

source of variation	formononetin (p value)	biochanin A (p value)	total isoflavone (p value)
site (S)	**<0.0001**	**<0.0001**	**<0.0001**
stand age (A)	**0.0488**	0.7662	0.8139
S × A	0.6463	**<0.0001**	**0.0006**
cultivar (C)	**0.0045**	**0.0251**	**0.0058**
A × C	0.3638	0.7892	0.6869
S × C	**0.0387**	0.2918	0.1731
S × A × C	0.3598	0.4025	0.3641
harvest (H)	0.4799	0.2807	0.3897
A × H	**0.0285**	0.1795	0.1161
S × H	**0.0044**	0.0787	**0.0157**
S × A × H	0.7805	**0.0232**	0.1734
C × H	0.0594	0.1378	0.0710
S × C × H	0.2116	0.1038	0.1578
A × C × H	0.9716	0.9897	0.8026
S × A × C × H	**0.0011**	**0.0002**	**0.0002**

[a] p values in bold were declared significant ($p < 0.05$).

80% water to 80% methanol and 20% water over the course of 30 min, following an initial 5 min of steady elution with 20:80% methanol/water. Isoflavones were detected at 254 nm (*24*, *25*). Purified chemical standards [formononetin and biochanin A, (Sigma−Aldrich, Mississauga, ON, Canada)] were used to identify isoflavones and determine their concentrations on a DM basis. The term "total isoflavone" in this study refers to the sum of formononetin and biochanin A concentrations, which together constituted approximately 97% of isoflavones detected in our studies.

Statistical Analyses. All data were subjected to analysis of variance (ANOVA) using the GLM procedure of the statistical analysis system (*26*) to identify significant treatment effects and interactions. Data from the cultivar evaluation experiment were analyzed in a combined analysis (*27*), regrouping sites, stand ages, cultivars, and harvests in a combined randomized complete block design with strip-plot restriction with cultivars and harvests as spatial and temporal strips, respectively (*28*). Stand ages, cultivars, and harvests were considered fixed effects, while the site was considered random. Appropriate F tests in each case were calculated following McIntosh (*27*). Data from the maturity and plant parts experiment were analyzed using a randomized complete block design with split-split-plot restriction with cultivars as the main plot, plant parts as split plots, and stage of maturity as split-split plots. Finally, for the preservation method experiment, data were analyzed using a randomized complete block design. In all experiments, differences between treatments were ascertained using least significant differences (LSDs) and F tests. Differences between treatments were declared at $p < 0.05$; only significant differences are discussed in the text.

RESULTS AND DISCUSSION

Cultivar Evaluation. The total isoflavone concentration ranged between 1525 and 16 756 μg g^{-1} of DM depending on cultivar, site, stand age, and harvest and averaged 8844 μg g^{-1} of DM with overall 55% formononetin. Isoflavone concentrations were affected by site, stand age, and cultivar main effects as well as interactions between these factors and harvest (**Table 1**). Variation across sites, stand ages, and harvests ranged from 82 to 248% for specific cultivars. However, results suggest that, despite environmental effects, differences in the isoflavone concentration between red clover cultivars are generally stable across environments and time and remain relatively unaffected by other factors evaluated.

Cultivars had the most effect on isoflavone concentrations. Averaged over site, stand age, and harvest, the total isoflavone concentration in the 10 cultivars evaluated varied by 43% ranging between 8923 and 12 753 μg g^{-1} of DM (**Table 2**).

Table 2. Isoflavone Concentration of 10 Red Clover Cultivars Grown in Sainte-Anne-de-Bellevue, QC, Canada[a]

cultivar	formononetin (μg g^{-1} of DM)	biochanin A (μg g^{-1} of DM)	total isoflavone (μg g^{-1} of DM)
AC Charlie	6137 ± 1105	4898 ± 1007	11 035 ± 2070
Azur	7421 ± 1119	5332 ± 1213	12 754 ± 2191
Belle	7070 ± 1161	5197 ± 1016	12 267 ± 2107
Concorde	7165 ± 1038	4714 ± 909	11 879 ± 1845
Prima	7198 ± 1150	5236 ± 1222	12 435 ± 2206
Ram	6811 ± 1623	4472 ± 1037	11 283 ± 2601
Scarlett	6863 ± 1165	4834 ± 727	11 696 ± 1768
Start	4842 ± 753	4081 ± 725	8923 ± 1269
Tempus	5997 ± 846	4356 ± 1071	10 353 ± 1849
Walter	6585 ± 1223	5144 ± 1333	11 729 ± 2380
$LSD_{0.05}$	951	669	1442

[a] Results present the cultivar main effect and are the average ± SD of two harvests done at two sites in 2 consecutive growing seasons for four replicates ($n = 32$).

One cultivar (i.e., "Start") consistently had the lowest isoflavone concentrations (4842, 4081, and 8923 μg g^{-1} of DM for formononetin, biochanin A, and total isoflavones, respectively) ranking 9th or 10th in total isoflavone concentration in all 8 site/stand age/harvest combinations. Differences between the other cultivars remained limited, averaging 23% for biochanin A and formononetin concentrations.

There was a four-way interaction between site, stand age, cultivar, and harvest for formononetin, biochanin A, and total isoflavone concentrations. This interaction reflects a three-way interaction between site, stand age, and harvest in 3 of the 10 cultivars evaluated, namely, AC Charlie (formononetin, biochanin A, and total isoflavone), Scarlett (biochanin A and total isoflavone), and Start (biochanin A). There was no site × age × cut interaction for the other 7 cultivars evaluated. The only other effect implicating cultivars was a cultivar × site interaction observed for formononetin. This interaction reflected that, while greater formononetin concentrations were observed for all cultivars in field B compared to field A, differences were significant only for five cultivars (i.e., AC Charlie, Concorde, Prima, Ram, and Start).

Site main effects were observed for all isoflavones. Overall, concentrations were 17, 30, and 22% greater in plants grown in field B than A for formononetin, biochanin A, and total isoflavone, respectively. Differences between sites might be attributed to inherent differences in soil types and characteristics between the two fields or differences in prevailing environmental conditions during experimentation. Soil fertility levels and several other abiotic factors including temperature, soil moisture levels, irradiance, and CO_2 concentration were all previously reported to affect isoflavone concentrations of other legumes (*29*−*31*). In addition to this main effect, the site was involved in interactions with stand age, harvest, and cultivar. The site × stand age interaction for biochanin A and total isoflavone concentrations is attributable to much greater seeding year concentrations in field B compared to field A. In the seeding year, plants grown in field B had 72 and 39% greater biochanin A and total isoflavone concentrations, respectively, than those grown in field A. No differences were observed between fields in the post-seeding year. A site × harvest interaction for formononetin and total isoflavone concentrations reflected the lower concentrations for the second harvest in field A, compared to all other site × harvest combinations.

Stand age overall had limited effects on isoflavone concentrations, mainly through interactions with other factors. A stand age main affect was observed only for formononetin, with its concentration being 15% higher in the post-seeding year than

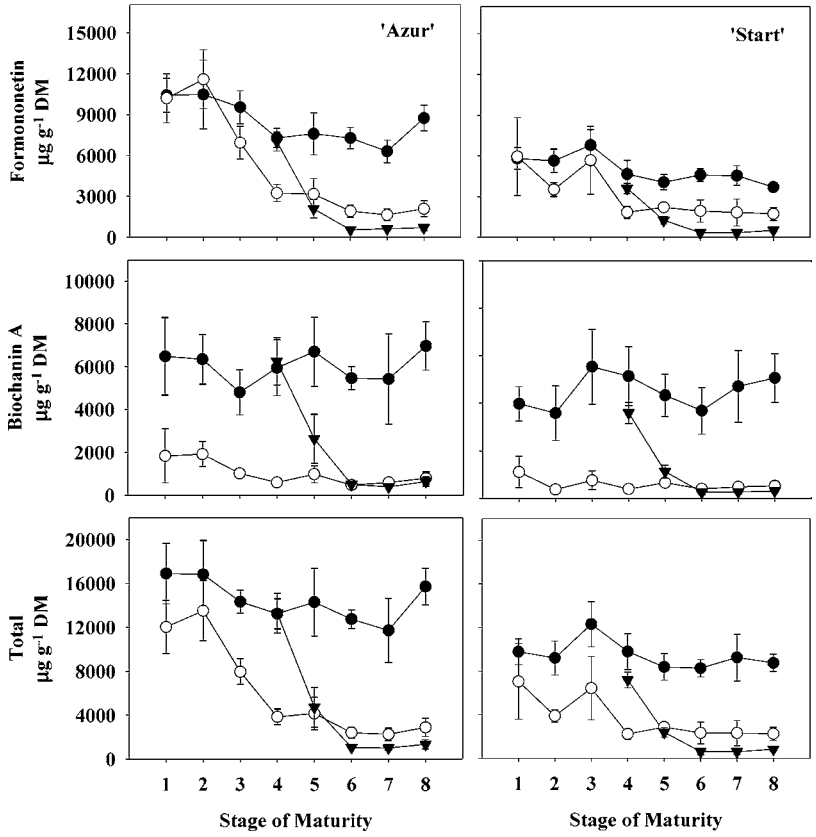

Figure 1. Isoflavone concentration in leaves (●), stems (○), and inflorescences (▼) of two field-grown red clover cultivars ("Azur" and "Start") sampled at eight different stages of maturity. Stage 1 (vegetative plants with three trifoliate leaves), 2 (vegetative plants with four trifoliate leaves), 3 (vegetative plants with five trifoliate leaves), 4 (inflorescence of main stem palpable), 5 (single buds on main stem discernible), 6 (at least one open flower on inflorescence of main stem), 7 (inflorescence of main stem at least halfway past flower), and 8 (inflorescence of main stem past flower and sepals still green). Data are the mean of four replicates ± SD.

in the seeding year when averaged across cultivars, sites, and harvests. A stand age × harvest interaction for formononetin reflected that differences between harvests differed depending on stand age. During the seeding year, across cultivars and sites, formononetin concentration dropped by an average of 38% (7571 to 4673 μg g^{-1} of DM) from the first to the second harvest, while in the post-seeding year, it rose by 11% (6710 to 7460 μg g^{-1} of DM). In addition, a three-way interaction between site, stand age, and harvest affected biochanin A concentration. The low biochanin A concentration at the first harvest of the post-seeding year in field B contributed strongly to this interaction. During the post-seeding year, biochanin A concentrations were similar in field A at both harvests (an average of 4624 μg g^{-1} of DM) but increased by 79% from the first to the second harvest in field B. No differences between harvests were observed at both sites in the seeding year.

Results suggest that despite strong environmental effects cultivar choice can impact isoflavone concentrations in red clover. Differences between cultivars will remain relatively constant at different sites, harvest, and for stands of different age. The cultivar "Start" distinguished itself from other cultivars containing low concentrations of both formononetin and biochanin A. While variation was observed among other cultivars, differences remained limited. Therefore, "Start" would be a cultivar of choice for producers aiming at reducing isoflavone intake of farm animals; options would be greater if red clover is to be produced as a source of isoflavone for the nutraceutical industry. The large site main effects and interactions between factors that we observed suggest that environmental factors will also have profound impacts on isoflavone concentrations in red clover, confirming similar observations made with other species (*17*, *25*, *32*). This underlines the need for further research to understand the impact of specific biotic and abiotic factors on red clover isoflavone concentration. Furthermore, it points out the need for nutraceutical manufacturers to regularly test purchased raw material, especially if using raw extracts or powdered plants, and reinforce the need for strict quality control practices.

Maturity and Plant Parts. Isoflavone concentrations were affected by the stage of maturity, plant part, and cultivar, as well as interactions between them. These interactions were largely due to changes, which differed depending on the isoflavone, of concentrations in leaves, stems, and inflorescences as plants mature, with the trend of change varying according to parts and cultivars. Relationships between plant parts and stage of maturity were although remarkably constant for both cultivars evaluated; differences in the magnitude of the response generated interactions implicating cultivars (**Figure 1**). Differences between cultivars were consistent with results of the cultivar evaluation experiment; when "Azur" was compared with "Start", "Azur" had 69, 44, and 59% greater formononetin, biochanin A, and total isoflavone concentrations, respectively, across stages of maturity and plant parts.

Across all stages of maturity and cultivars, average isoflavone concentrations were greatest in leaves (11 970 μg g^{-1} of DM), intermediate in stems (4896 μg g^{-1} of DM), and lowest in inflorescences (3297 μg g^{-1} of DM). Differences between plant parts, however, varied significantly depending on the stage of maturity (**Figure 1**). Before the onset of flowering (stages 1−3), there was little or no difference in leaf and stem formononetin concentration for both cultivars. The concentration of biochanin A was none the less 3−8 times greater in leaves than stems,

with greater differences observed with "Start", for which differences were also reflected in total isoflavone concentrations. In the earliest stage of flowering (stage 4), leaves and inflorescences had comparable isoflavone concentrations, which were 3 times greater than in stems. However, as flowering progressed, formononetin and total isoflavone concentrations differed in the three parts, being greatest in leaves, intermediate in stems, and lowest in inflorescences, with as much as an 11-fold difference observed between leaves and inflorescences. The biochanin A concentrations of stems and inflorescences were similar and were only 9% of that found in leaves.

While change with maturity in isoflavone concentrations of leaves and stems differed depending on the isoflavone, trends for inflorescences were similar for all isoflavones (**Figure 1**). Concentrations of formononetin, biochanin A, and total isoflavone in inflorescences were high at the onset of flowering but decreased sharply by an average of 92% by the next stage, to later stabilize. Leaves and stems had their highest formononetin concentration at early maturity stages, with a gradual decrease observed until the onset of flowering. At that point, the formononetin concentrations in leaves and stems were 75 and 32%, respectively, of those observed at the first stage sampled. Upon the initiation of flowering, formononetin concentrations in leaves and stem stabilized, with no difference observed among later stages, except for an increase in leaves at the last stage sampled (stage 8, inflorescence of the main stem past flower with sepals still green). The biochanin A concentration was not affected by the stage of maturity in leaves, but in stems, concentrations were slightly greater before the onset of flowering than all subsequent stages.

This experiment illustrates that, as plant maturity progresses, variations in isoflavone concentrations depend on the plant part. Concentrations of all isoflavones decreased sharply in stems and flowers, while a smaller decrease, only in formononetin, was observed in leaves. These differences in trends resulted in leaves having much greater isoflavone concentrations than either flowers or stems by the time flower buds have become visible. These results are generally in agreement with the results of a greenhouse experiment reported by McMurray et al. (*7*). In both studies, formononetin concentration decreased from early harvests during the vegetative stage through late flower in both leaves and stems. Interestingly, both experiments also observed an increase in formononetin concentration in leaves at approximately the onset of flower desiccation. Differences between studies were observed; for example, we observed that stems initially had comparable formononetin concentrations as leaves, while in the first two samplings in the experiment performed by McMurray et al. (*7*), leaves had higher concentrations even then. It is difficult to accurately compare studies because stages given by McMurray et al. (*7*) are only vaguely described. Our results are also in accordance with Wu et al. (*9*), who reported that, when averaged over red clover plants collected once in four different environments, total isoflavones were greatest in leaves, intermediate in stems, and lowest in flowers. Variation was although observed between plants collected in different environments; these could be explained by the fact that plants might have been harvested at different stages of maturity.

However, the results that we observed are in contrast to those reported by Vetter (*8*), who found that flowers had higher concentrations of biochanin A and slightly higher concentrations of formononetin than did leaves. It is difficult to confidently compare between Vetter (*8*) and our study, because the plant material used for analysis in Vetter (*8*) is simply reported as being collected during the "flowering stage" and, as can be seen in the current study, isoflavone levels can vary significantly during the growth of red clover. However, it can be assumed that the results are in sharp contrast, nonetheless, because flowers in our study were found to have much lower concentrations of both formononetin and biochanin A by the time buds are distinctly visible, well before the coloring of flowers has occurred.

Table 3. Isoflavone Concentration of Fresh Red Clover (28% DM) or Red Clover Preserved as Silage (42% DM) or Hay (90% DM)[a]

treatment	formononetin (μg g^{-1} of DM)	biochanin A (μg g^{-1} of DM)	total isoflavone (μg g^{-1} of DM)
fresh	9021 ± 366	5443 ± 682	14 465 ± 1021
silage	7221 ± 548	4980 ± 611	12 201 ± 1154
hay	6468 ± 155	5136 ± 286	11 604 ± 402
$LSD_{0.05}$	569	NS[b]	1480

[a] Data are the mean of four replicates ± SD. [b] NS, means are not significantly different ($p > 0.05$).

The low isoflavone concentrations that we observed in inflorescences are of importance for the nutraceutical industry, because manufacturers are currently often only using flowers for the extraction of red clover isoflavone (*15*). This study suggests that this strategy may be inappropriate and needs to be re-evaluated, especially given the difficulty of harvesting flowers only, which requires manual labor, increasing production costs. The use and harvest of vegetative material would maximize the isoflavone concentration of red clover. Our results, however, indicate that current recommendations for the harvest of red clover as a source of forage for ruminants, which are to harvest herbage at early flowering stages, would minimize isoflavone concentrations. Harvesting red clover at this stage may slightly reduce the adverse affect that isoflavones have on animal reproduction.

Preservation Method. Differences were observed between preservation methods evaluated for formononetin and total isoflavone concentrations (**Table 3**). The total isoflavone concentration was 22% higher in fresh material (14 464 μg g^{-1} of DM) than either silage or hay (12 200 and 11 604 μg g^{-1} of DM, respectively), while the formononetin concentration was highest in fresh material, intermediate in silage, and lowest in hay (9021, 7220, and 6468 μg g^{-1} of DM, respectively). No differences were observed between treatments in the biochanin A concentration. Conversely, Sarelli et al. (*16*) found isoflavone concentrations to be 18% greater in ensiled red clover than in wilted herbage before ensiling. Differences between studies could be due to differences in the ensiling process. Ensiling is a dynamic process, dependent on many factors, including initial herbage composition, microbial population, pH, and temperature, all of which fluctuate throughout the ensiling process (*33*). These factors were not measured in our experiment; it is possible that any or all of these factors have an impact on the isoflavone concentration in red clover silage. The effect that the preservation method has on the red clover isoflavone concentration is important for producers looking to control isoflavone intake of sheep and cattle. On the basis of our results, feeding hay, rather than fresh herbage, could reduce risks associated with red clover because hay has a lower formononetin content. In the rumen, formononetin is converted to equol, which is much more estrogenic, while biochanin A is broken down to nonestrogenic *para*-ethyl phenol (*34*). Therefore, lowering the formononetin concentration would contribute most in lowering estrogenicity of red clover.

In conclusion, the present study suggests that environmental factors have a large effect on isoflavone concentrations of red clover. Cultivars consistently differ in their isoflavone concentration, with one of them ("Start") having especially low

concentrations. Specific cultivar recommendations could thus be made depending on if concentrations are to be maximized or minimized depending on the intended use. The large differences observed between plant parts and stages of maturity underline the need to elaborate new recommendations specific for the production of red clover used for isoflavone extraction. The use of foliage from plants before the onset of flowering should be considered as an alternative to the current predominant practice of using flowers. In the case of red clover to be fed to ruminant animals, current recommendations and practices appear appropriate if producers intend to minimize isoflavone consumption by animals. However, producers may want to consider, when possible, feeding red clover hay rather than fresh herbage to minimize possible reproductive problems.

LITERATURE CITED

(1) Cassidy, A.; Bingham, S.; Setchell, K. Biological effects of isoflavonoids in young women: Importance of the chemical composition of soyabean products. *Br. J. Nutr.* **1995**, *74*, 587−601.

(2) Nestel, P.; Cehun, M.; Chronopoulos, A.; DaSilva, L.; Teede, H.; McGrath, B. A biochanin-enriched isoflavone from red clover lowers LDL cholesterol in men. *Eur. J. Clin. Nutr*. **2004**, *48*, 403−408.

(3) Jarred, R. A.; Keikha, M.; Dowling, C.; McPherson, S. J.; Clare, A. M.; Husband, A. J.; Pedersen, J. S.; Frydenberg, M.; Risbridger, G. P. Induction of apoptosis in low to moderate-grade human prostate carcinoma by red clover-derived dietary isoflavones. *Cancer Epidemiol., Biomarkers Prev*. **2002**, *11*, 1689−1696.

(4) Atkinson, C.; Compston, J. E.; Day, N. E.; Dowsett, M.; Bingham, S. A. The effects of phytoestrogen isoflavones on bone density in women: A double-blind, randomized, placebo-controlled trial. *Am. J. Clin. Nutr*. **2004**, *79*, 326−333.

(5) van de Wiejer, P. H. M.; Barentsen, R. Isoflavones from red clover (Promensil) significantly reduce menopausal hot flush symptoms compared with placebo. *Maturitas* **2002**, *42*, 187−193.

(6) Dedio, W.; Clark, K. W. Biochanin A and formononetin content in red clover varieties at several maturity stages. *Can. J. Plant Sci.* **1968**, *48*, 175−181.

(7) McMurray, C. H.; Laidlaw, A. S.; McElroy, M. The effect of plant development and environment on formononetin concentration in red clover (*Trifolium pratense* L.). *J. Sci. Food Agric*. **1986**, *37*, 333−340.

(8) Vetter, J. Isoflavones in different parts of common *Trifolium* species. *J. Agric. Food Chem.* **1995**, *43*, 106−108.

(9) Wu, Q.; Wang, M.; Simon, J. E. Determination of isoflavones in red clover and related species by high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. *J. Chromatogr., A* **2003**, *1016*, 195−209.

(10) Institute for Environment and Health. *Phytoestrogens in the Human Diet*; http://www.le.ac.uk/ieh/webpub/webpub.html (accessed March 2005), 2000.

(11) Beck, A. B. The oestrogenic isoflavones of subterranean clover. *Aust. J. Agric. Res.* **1964**, *15*, 223−230.

(12) Braden, A. W. H.; Shutt, D. A. The metabolism of pasture oestrogens in ruminants. *Proc. 11th Int. Grassland Congr*. **1970**, 770−773.

(13) Moorby, J. M.; Fraser, M. D.; Theobald, V. J.; Wood, J. D.; Haresign, W. The effect of red clover formononetin content on live-weight gain, carcass characteristics, and muscle equol content of finishing lambs. *Anim. Sci.* **2004**, *79*, 303−313.

(14) Wong, E. Isoflavone contents of red and subterranean clovers. *J. Sci. Food Agric.* **1963**, *14*, 376−379.

(15) Filière des Plantes Médicinales Biologique du Québec. In *Le Trèfle Rouge: Guide de Production Sous Régie Biologique*; Filière des Plantes Médicinales Biologique du Québec: Sherbrooke, QC, Canada, 2004.

(16) Sarelli, L.; Tuori, M.; Saastamoinen, I.; Syrjala-Qvist, L.; Saloniemi, H. Phytoestrogen content of birdsfoot trefoil and red clover: Effects of growth stage and ensiling method. *Acta Agric. Scand., Sect. A* **2003**, *53*, 58−63.

(17) Smith, G. R.; Randel, R. D.; Bradshaw, C. Influence of harvest date, cultivar, and sample storage method on concentration of isoflavones in subterranean clover. *Crop Sci.* **1986**, *26*, 1013−1016.

(18) CRAAQ (Centre de Référence en Agriculture et Agroalimentaire du Québec). In *Guide de Référence en Fertilisation*, 1st ed.; Centre de Référence en Agriculture et Agroalimentaire du Québec: Sainte-Foy, QC, Canada, 2003.

(19) Ohlsson, C.; Wedin, W. F. Phenological staging schemes for predicting red clover quality. *Crop Sci.* **1989**, *29*, 416−420.

(20) Seguin, P.; Mustafa, A. F. Chemical composition and ruminal nutrient degradabilities of fresh and ensiled Kura clover (*Trifolium ambiguum* M. B.). *Can. J. Anim. Sci.* **2003**, *83*, 577−582.

(21) Pettersson, H.; Heinz-Kiessling, K. Liquid chromatographic determination of the plant estrogens coumestrol and isoflavones in animal feed. *J. Assoc. Anal. Chem.* **1984**, *47*, 503−505.

(22) Edwards, R.; Tiller, S. A.; Parry, A. D. The effect of plant age and nodulation on the isoflavonoid content of red clover (*Trifolium pratense*). *Plant Physiol.* **1997**, *150*, 603−610.

(23) Toebes, A. H. W.; de Boer, V.; Verkleu, J. A. C.; Lingeman, H.; Ernst, W. H. O. Extraction of isoflavone malonylglucosides from *Trifolium pratense* L. *J. Agric. Food Chem.* **2005**, *53*, in press.

(24) Wang, C.; Sherrard, M.; Pagadala, S.; Wixon, R.; Scott, R. A. Isoflavone content among maturity group 0 to II soybeans. *J. Am. Oil Chem. Soc*. **2000**, *77*, 483−487.

(25) Seguin, P.; Zheng, W.; Smith, D. L.; Deng, W. Isoflavone content of soybean cultivars grown in eastern Canada. *J. Sci. Food Agric.* **2004**. *84*, 1327−1332.

(26) Statistical Analysis Systems Institute. *SAS User's Guide: Statistics*, SAS Institute Inc.: Cary, NC, 1989.

(27) McIntosh, M. S. Analysis of combined experiments. *Agron. J.* **1983**, *75*, 153−155.

(28) Gomez, K. A.; Gomez, A. A. In *Statistical Procedures for Agricultural Research*, 2nd ed.; Wiley and Sons: New York, 1984.

(29) Rossiter, R. C.; Beck, A. B. Physiological and ecological studies on the oestrogenic isoflavones on subterranean clover (*T. subterraneum* L.) II. Effects of phosphate supply. *Aust. J. Agric. Res.* **1966**, *17*, 447−456.

(30) Bennett, J. O.; Yu, O.; Heatherly, L. G.; Krishnan, H. B. Accumulation of genistein and daidzein, soybean isoflavones implicated in promoting human helath, is significantly elevated by irrigation. *J. Agric. Food Chem.* **2004**, *52*, 7574−7579.

(31) Caldwell, C. R.; Britz, S. J.; Mirecki, R. M. Effect of temperature. Elevated carbon dioxide, and drought during seed development on the isoflavone content of dwarf soybean [*Glycine max* (L.) Merill] grown in controlled environments. *J. Agric. Food Chem*. **2005**, *53*, 1125−1129.

(32) Seguin, P.; Zheng, W. Phytoestrogens content of alfalfa cultivars grown in eastern Canada. *J. Sci. Food Agric.* **2005**, manuscript submitted.

(33) Charmley, E. Towards improved silage quality−A review. *Can. J. Anim. Sci*. **2001**, *81*, 157−168.

(34) Cox, R. I.; Braden, A. W. H. The metabolism and physiological effects of phyto-oestrogens in livestock. *Proc. Aust. Soc. Anim. Prod.* **1974**, *10*, 122−129.

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