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Red Clover *Trifolium pratense* L. Phytoestrogens: UV-B Radiation Increases Isoflavone Yield, and Postharvest Drying Methods Change the Glucoside Conjugate Profiles

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Isoflavone extracts of red clover *Trifolium pratense* L. (cv. Pawera) with dissimilar glucoside conjugate profiles were obtained by employing different postharvest drying methods. The most prominent isoflavones found were formononetin and biochanin A and their corresponding glucosides and malonyl glucoside esters. Postharvest freeze drying inhibited the conversion of the glycosides to the aglycones, while vacuum drying allowed for maximum conversion of the glycosides to their corresponding aglycones. Air drying produced a low level of the aglycones formononetin and biochanin A, and oven drying promoted decarboxylation of the malonyl glucosides to the acetyl glucosides. Exposure to enhanced UV-B radiation resulted in an increase in total formononetin and biochanin A isoflavone levels, indicating that harvest during a period of high ambient UV-B radiation may be appropriate for maximum yield. The levels of caffeic acid and flavonols also increased by about 40 and 250%, respectively, on exposure to enhanced UV-B radiation.

KEYWORDS: ***Trifolium pratense*; red clover; phytoestrogens; isoflavones; UV-B; postharvest; HPLC**

INTRODUCTION

The phenylpropanoid pathway is perhaps the best characterized of all metabolic pathways in plants, and many of its flavonoid products are now being used as health supplements in humans. The isoflavones in particular have estrogenic properties and have recently gained a considerable reputation as phytoestrogen therapeutic agents in human health (*1*). Phytoestrogens are frequently associated with prevention of breast and prostate cancer and are also believed to be preventative against cardiovascular disease (*2*−*4*).

There are a number of dietary phytoestrogen supplements marketed for conditions such as postmenopausal symptoms, and assessment of some of these for their estrogenic activity is described in the literature (*5*−*8*). The predominant ingredient in some supplements is an extract of red clover, *Trifolium pratense* L. Red clover is a rich natural source of the isoflavones daidzein, genistein, formononetin, and biochanin A, which occur in the plant as the glucosides, glucoside malonate esters, or free aglycones (**Figure 1**). The plant is able to store the more soluble glucosides and glucoside malonate esters in the vacuole. The identification and analysis of the various isoflavonoids and general flavonoids found in red clover is normally conducted using high-performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LCMS) (*5*−*12*).

When red clover is grown specifically for the extraction of isoflavones, it is important to optimize preharvest growth parameters and to establish postharvest protocols, to maximize yields. It is well-known that introduction of environmental stress to plants causes an upregulation of the phenylpropanoid pathway (*13*, *14*). In particular, exposure to ultraviolet-B radiation (UV-B) results in a rapid and coordinated increase in gene expression, protein levels, and enzyme activities of the phenylpropanoid biosynthetic pathway, resulting in increases in the concentration of flavonols, anthocyanins, and isoflavones within the leaves of plants (*15*). As a result, when nine populations of white clover *Trifolium repens* L. were exposed to UV-B, there was a significant increase in the levels of the flavonol glycosides of quercetin and kaempferol (*16*). In another study on *Trifolium repens* L., UV-B increased the levels of UV-B-absorbing compounds and this effect was synergistically enhanced by water stress (*17*). Because isoflavones are part of the phenylpropanoid pathway, it is likely that natural isoflavone levels could be similarly enhanced and should be at a maximum in plants during midsummer when ambient levels of UV-B are at their highest.

Very little has been published regarding the comparative bioavailability and pharmacokinetics of the red clover isoflavone aglycones, glucosides, and glucoside malonyl esters (*18*). It is largely believed that it is only the aglycones that are absorbed from the human intestine; hence, hydrolysis of the glycosides and their conjugates to the aglycones is the essential first step in the bioavailability of isoflavones in humans (*19*). In plants such as alfalfa, microbial infection of the plant is known to

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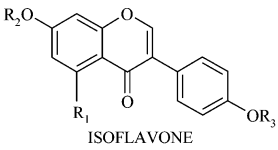
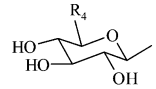


ISOFLAVONE



R_4=H: glucosyl
R_4=$COCH_3$: acetylglucosyl
R_4=$COCH_2COOH$: malonylglucosyl

	R_1	R_2	R_3
Formononetin glucoside, **(5)**	H	glucosyl	CH_3
Daidzein, **(6)**	H	H	H
Formononetin malonylglucoside, **(7)**	H	malonylglucosyl	CH_3
Biochanin A glucoside, **(8)**	OH	glucosyl	CH_3
Formononetin acetylglucoside, **(9)**	H	acetylglucosyl	CH_3
Biochanin malonylglucoside, **(10)**	OH	malonylglucosyl	CH_3
Formononetin, **(11)**	H	H	CH_3
Biochanin A acetylgluoside, **(12)**	OH	acetylglucosyl	CH_3
Biochanin A, **(13)**	OH	H	CH_3

Figure 1. Structures of major isoflavones.

induce hydrolysis of the glucosides to produce the free aglycones (*20*). In freshly harvested and crushed vegetable material, enzymic hydrolysis of many flavonoid glycosides occurs very readily in the presence of heat and moisture (*9*). It is likely therefore that postharvest protocols as well as growing conditions could have significant effects on the extent of enzymic hydrolysis of red clover isoflavone glycosides.

In this study, the influence of postharvest drying regimes and the effects of UV-B radiation on the levels and nature of the isoflavones formononetin and biochanin A in red clover *Trifolium pratense* L. (cv. Pawera) were investigated. Red clover plants were subjected to enhanced UV-B, ambient UV-B, and very low UV-B radiation during early plant growth. Four different drying techniques of the harvested material, including air, vacuum, freeze and oven drying, were examined. The aqueous methanolic extracts of the dried material were analyzed by HPLC for isoflavone content. Other flavonoids may play a role in the protection of plants from UV-B-induced damage (*14*), and for this reason, the levels of flavonols and caffeic acids were included in this study.

MATERIALS AND METHODS

Plant Growth Chamber. Plants were grown in an outdoor plastic-covered chamber, which was divided into three sections providing enhanced ultraviolet-B radiation (+UV-B), ambient UV-B, and very low UV-B. The low UV-B (−UV-B) region was clad in Mylar, which allows approximately 5% ambient UV-B to penetrate into this region of the chamber. The cladding material over the +UV-B and ambient regions (Teflon FEP) was transparent to solar UV-B. Enhanced UV-B radiation was provided by eight 40 W UV-B fluorescent tubes type 313 (Q-Panel), while the adjacent ambient and −UV-B regions had dummy tubes in similar orientation to provide equivalent shading. The UV-B illumination system was continuously adjusted to provide a constant enhancement of UV-B over ambient levels throughout the day regardless of overhead conditions (*21*, *22*). This was achieved by continuously monitoring the ambient UV-B radiation in the ambient region of the growth chamber and modulating the brightness of the UV-B lamps in the enhanced region to provide UV-B radiation, enhanced approximately 25% over ambient at this time of the year. Ultraviolet-B radiation was recorded continuously in the enhanced and ambient UV-B regions of the chamber using Actinic UV radiometers consisting of a diffuser, an interference filter, and a type SED240/ACTS270/W solar blind phototube (International Light, Inc., Newburyport, MA) (*23*). Millivolt readings were recorded automatically, averaged each 10 min, and then integrated for 6 am−6 pm, for each day to provide an arbitrary scale of daily total UV-B dose. Ambient UV-B levels were typical of those of mid to late summer in New Zealand at this time (*23*).

Plant Material. Seeds of red clover, *Trifolium pratense* (cv. Pawera), were sown directly onto trays of moist potting mix, covered with 2−3 mm of sieved potting mix, and allowed to germinate under low light indoors. After 1 week, the trays were placed in the plastic house in the −UV-B region. After a further week of hardening off, the trays were distributed to the three UV-B treatment regions of the plastic house and watered to excess using an automatic watering system. The temperature within the growth chamber was maintained within ±1 °C of ambient outside temperatures throughout the period of the study by fans, which continuously drew outside air into the chamber. A total of 3 weeks later when plants were approximately 100 mm tall, individual mature fully unfolded leafletss without petioles were cut from the uppermost part of several plants using a razor blade (3−5 leaves total) and processed together as one sample. A total of 5−7 samples were collected in this way on February 21st, 2001, for each of the drying treatments below and for each UV-B treatment when appropriate. Leaflets were randomly assigned to treatments. In later collections in March and early April, the same plants were used, although leaflets of a similar age to those sampled in February were chosen, from similar locations on the plants. None of the plants were in flower at any stage during the experiments.

Air Drying. Samples from the ambient UV-B region were laid on paper towels in the plastic growth house in the same region as they were grown and allowed to air-dry (12−24 h). Samples were collected and stored in airtight containers for later extraction.

Oven Drying. Samples from the ambient UV-B region were quickly transferred to a hot oven (100 °C) within 10 min of being cut from the plants. Drying took approximately 3 h, and samples were then collected and stored in airtight containers for later extraction.

Vacuum Drying. Samples from all three UV-B treatments were immediately frozen in liquid nitrogen within 20 s of being cut from the plant. They were stored briefly in liquid nitrogen, and then transferred to a −80 °C freezer overnight. The frozen samples were then transferred to a large desiccator and a rotary pump vacuum was applied, while the samples were still frozen. The samples were allowed to warm to room temperature under vacuum, a process that took approximately 30−60 min. Vacuum drying of the samples was complete after 48 h.

Freeze Drying. Samples were collected on two separate occasions: March 11th, 2001, from the ambient region, and on April 4th, 2001, from all three UV-B treatment regions. They were frozen immediately in liquid nitrogen and finely crushed using a steel rod and stored overnight in liquid nitrogen. Freeze-drying was performed using a block of brass 75 mm in diameter and 100 mm high, with 5 holes 10 mm in diameter × 20 mm deep in the upper surface. The block was cooled to liquid nitrogen temperature, and the holes were filled with liquid nitrogen. The crushed samples were transferred to the holes in the brass block and maintained at liquid nitrogen temperature. The brass block was then placed in a vacuum chamber supported on three fine steel pins to minimize thermal contact, and a rotary pump vacuum was applied. The vacuum chamber was shielded from radiant heat by an additional cover. Under these conditions, the brass block and samples took approximately 10−12 h to warm to room temperature, by which time the samples were dry. They were then transferred to airtight containers and stored for later extraction.

Isoflavone Extraction and HPLC Analysis. Approximately 100 mg of dried and finely ground leaves were extracted under occasional vortex shaking for 12 h in 80% methanol (5 mL), followed by centrifugation and filtration. A 10 μL sample was injected directly for HPLC analysis. Analytical HPLC was conducted using a Waters 600E solvent delivery system, Waters 996 diode array detector, and Jasco

851-AS intelligent sampler, and results were analyzed using Waters Millennium 2010 software. A 119 mm × 4 mm i.d., 4 μm Supersphere LiChroCART 125-4 RP-18 end-capped column was used (Merck) with a gradient solvent system comprising solvent A (5% HCOOH) and solvent B (CH_3CN) mixed using a linear gradient starting with 10% B, increasing to 20% B at 10 min, 40% B at 30 min, 80% B at 33 min, 95% B at 37 min, and 100% B at 39 min, followed by re-equilibration of the column. Isoflavones, caffeic acid, and flavonols were detected at 260, 320, and 352 nm, respectively, with the derivative peaks being identified on the basis of the on-line spectrum recorded for each identifiable peak. The identity of the isoflavones was confirmed by LCMS−electrospray ionization with a Mariner 5158, high resolution, time-of-flight instrument (Applied Biosystems) operated in the positive mode. Total levels were calculated by adding the integrated areas of all of the respective and appropriate peaks, and the result was compared to a calibration curve prepared using biochanin A (isoflavones), caffeic acid, and rutin (flavonols). Isoflavone levels were expressed as aglycone equivalents and calculated by adjusting for molecular-weight differences of the individual conjugates. Total formononetin and biochanin A isoflavones were calculated as the sum of the glucoside, glucoside malonyl and acetyl esters, and the free aglycone forms and expressed as aglycone equivalents.

Statistical Analysis. Comparisons between means were performed using Student's *t*-test statistic.

RESULTS AND DISCUSSION

Effect of Postharvest Drying on the Isoflavone Glucoside Conjugate Profile. Red clover is a rich source of isoflavones and is becoming increasingly grown on a commercial scale specifically for dietary phytoestrogen supplements (*5*). While isoflavones are sometimes extracted in laboratory conditions without drying the sample (*24*), commercial postharvest procedures require drying the crop before extraction to avoid deterioration. Leaves harvested from plants grown under ambient UV-B conditions were therefore dried using four different methods, and HPLC analyses were performed to determine flavonoid concentrations. The HPLC chromatograms (**Figure 2**) detected at 260 nm showed that the method of drying had a significant influence on the glucoside conjugate profile. The dominant isoflavones detected were formononetin and biochanin A and their respective glucosides and malonyl/acetyl glucosides.

The chromatogram of the freeze-dried harvest (**Figure 2**) showed prominent peaks (7 and 10) for the malonyl glucoside esters of formononetin and biochanin A and lesser peaks (5 and 8) for the two respective glucosides. The aglycones formononetin and biochanin A were present in freeze-dried material in trace amounts less than 1% of the total isoflavones yield. β-Glucosidases are present in red clover tissue, and therefore, isoflavone glucosides may be readily hydrolyzed to their corresponding aglycones (*9*, *24*). In our freeze-drying method, postharvest enzymatic hydrolysis was prevented and formononetin and biochanin A occurred in the plant tissue predominantly as their malonyl glucoside esters. This is because freeze-dried samples were plunged into liquid nitrogen immediately after excision from the plant, and the leaf material did not warm to ambient temperatures until it was completely dry. In our hands, our relatively small samples warmed within hours when using a standard laboratory freeze drier, and the samples became hydrated. We therefore recommend that this sort of instrument should not be used for freeze drying of small specimens. Under our freeze-drying regime, there was no liquid-water phase for enzymatic degradation.

The low concentration of aglycones in freeze-dried material was evident in all five samples collected from the ambient UV-B region on March 11th ($n = 5$) as shown in **Figure 2**. Furthermore, this relative composition was unaltered in samples collected nearly a month later on April 4th nor was it affected by UV-B radiation. These later samples, collected from the −UV-B ($n = 7$), ambient UV-B ($n = 8$), and +UV-B ($n = 8$) regions all had less than 1% aglycones (0.65, 0.85, and 0.58%, respectively). These observations demonstrate that isoflavone glycosides predominate in the clover tissue and that the ratio of glycoside/aglycone is not affected by the age of the plant at sampling time, the time of the year, nor the levels of UV-B irradiation. We therefore suggest that this high glycoside makeup was the natural composition of our clover leaves, and this did not change over the period of the study. **Figure 3** illustrates ratios of total glycosides to aglycones (malonylglucosides and glucosides) utilizing this method and shows that other drying methods resulted in different mixtures of these compounds. Our freeze-drying method allows for extraction of isoflavone compounds from clover leaves in a condition closest to their natural state.

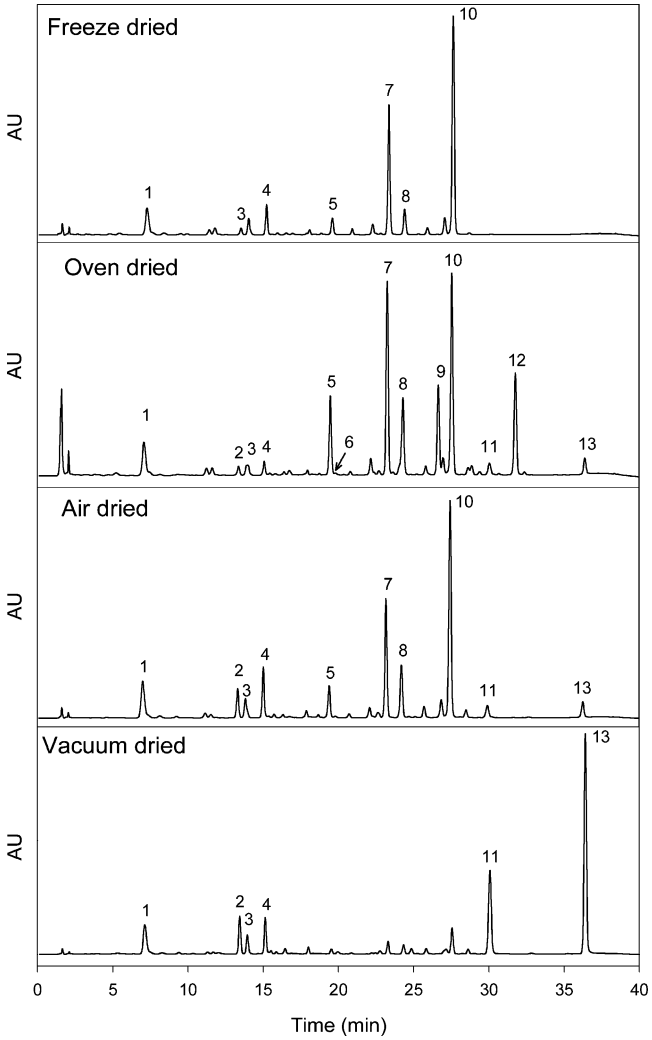


Figure 2. HPLC chromatograms detected at 260 nm, showing the effect of different drying methods on isoflavone glycoside conjugate profiles. Note that the freeze-dried sample was collected later in the season. Peak 1, caffeic acid; peaks 2–4, flavonols; peak 5, formononetin glycoside; peak 6, daidzein; peak 7, formononetin malonate glycoside; peak 8, biochanin-A glycoside; peak 9, formononetin acetyl glycoside; peak 10, biochanin A malonate glycoside; peak 11, formononetin; peak 12, biochanin-A acetyl glycoside; peak 13, biochanin-A.

The molar ratio of the formononetin and biochanin malonyl glucosides to the corresponding glucosides was 5.3:1 and 6.2:1, respectively. Toebes et al. (*24*) and Lin et al. (*11*) also

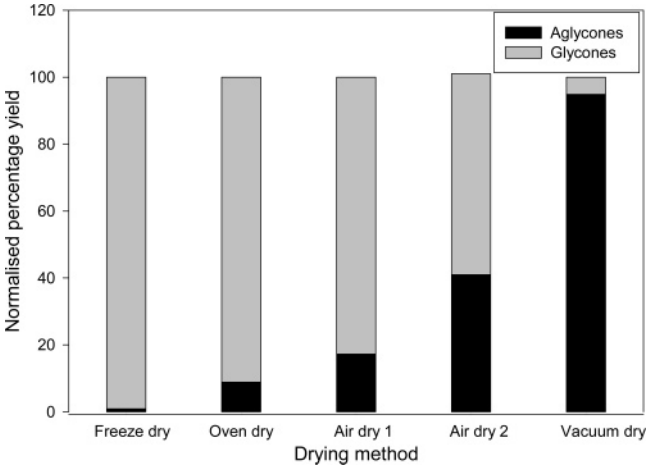


Figure 3. Effect of the drying method on formononetin and biochanin-A glycoside and aglycone composition. The vacuum-dried and oven-dried samples were collected on February 21st, 2001. The two air-dried samples were collected on February 21st (1) and April 4th (2), and the freeze-dried samples were collected on March 11th. Note that comparisons between samples collected at different times should be interpreted with caution. The sample size for each treatment was as follows: freeze dry ($n = 8$), oven dry ($n = 6$), air dry 1 ($n = 7$), air dry 2 ($n = 7$), and vacuum dry ($n = 7$).

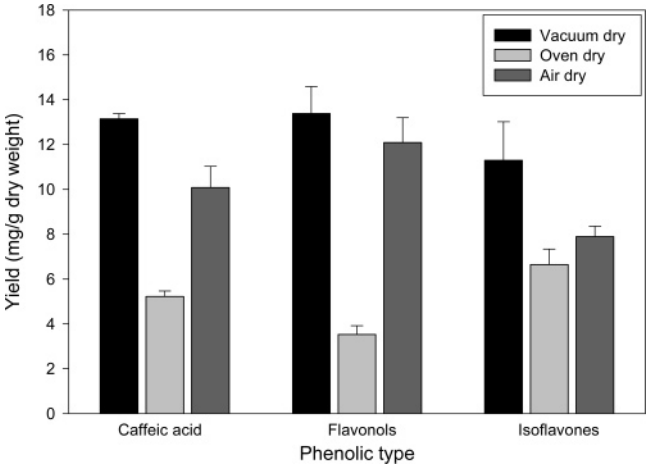


Figure 4. Effect of the drying method on the yield of total caffeic acids, flavonols, and isoflavones (formononetin and biochanin-A, expressed as aglycone equivalents). Data shown are mean and standard error. Sample size was $n = 7$ for all data points, except oven dried isoflavones, where $n = 6$.

observed that malonylated glucosides were the most dominant isoflavones in red clover leaves.

The total formononetin and biochanin A isoflavones (sum of all conjugate forms) obtained for freeze-dried material was 5.88 mg/g of dw (expressed as aglycone equivalents). Note that the material for the other postharvest drying treatments was collected nearly 3 weeks earlier (February 21st) than this freeze-drying material (March 11th). Here, our discussion of freeze-dried material has been restricted to comparisons of relative concentrations of isoflavones conjugates and not their absolute concentrations. The freeze-dry yields of total flavonoids are therefore not directly comparable with other drying treatments and are not included in subsequent figures. The concentration of total isoflavones of further clover samples collected and freeze-dried on April 4th was similar to the March 11th samples (data not shown).

Vacuum drying resulted in almost complete conversion of glucosides to aglycones, with major peaks being observed for formononetin and biochanin A in the HPLC chromatogram (**Figure 2**). With this method, the excised leaf material warmed to room temperature (and to a fully hydrated state) within 30−60 min of being placed under vacuum. Complete drying took several days and allowed enzymatic conversion of 95% of the isoflavone glucosides to their aglycones (**Figure 3**). The total formononetin and biochanin A yield from samples collected on February 21st was 11.29 mg/g of dw (**Figure 4**). This concentration was the highest obtained in all treatments ($p < 0.05$) and may be due to the freezing and thawing process employed, which would have broken vacuole and cell membranes. Once cell contents were released in this manner, perhaps the flavonoid compounds were more accessible for subsequent extraction.

Malonyl glucosides of formononetin and biochanin A were the prominent isoflavones, comprising 22 and 41% of the total, respectively, in the air-dried harvest collected on February 21st. The total malonyl glycoside yield of formononetin and biochanin A expressed as aglycone equivalents was 7.89 mg/g of dw (**Figure 4**), which was significantly lower than for vacuum-dried material ($p < 0.05$). Low peaks for formononetin and biochanin A appeared in the HPLC chromatogram, indicating that some hydrolysis had occurred. The respective ratios obtained for malonyl glucosides/glucosides/aglycones for formononetin were 2.9:1:1 and for biochanin A were 3.2:1:0.8. The ratios for malonylated glucosides to their respective glucosides have reduced from that observed in the freeze-dried samples, and this may reflect a conversion of malonylated glucosides to their glucosides and/or their aglycone equivalents. The percentage of total aglycones for this method in late summer was 17% (**Figure 3**). A second air-drying harvest was made later in the season on April 4th. The total concentration of isoflavones was similar to the earlier measurement at 7.1 mg/g of dw, but the percentage of total aglycones was significantly higher at 41% ($p < 0.01$). At this time of the year, the ambient temperatures were lower than for the first harvest, and therefore, drying of the plant material took longer, perhaps providing a more extended period in a relatively hydrated state for enzymatic hydrolysis of the samples to occur.

The yield of total formononetin and biochanin A isoflavones obtained from the oven-dried material was 6.64 mg/g (**Figure 4**), which was significantly less ($p < 0.02$) than that for vacuum-dried material. Oven drying resulted in heat-induced decarboxylation of the malonyl glucosides to produce the acetyl glucosides of formononetin and biochanin A. The ratios obtained for malonyl glucosides/acetyl glucosides/glucosides/aglycones for formononetin were 1.79:1.42:1:0.42 and for biochanin A were 1.79:1.36:1:0.39. The relatively high temperature of the oven may have induced postharvest degradation of the caffeic acid and the flavonols, producing considerably lower yields than the vacuum-dried samples (**Figure 4**); however, the yield for the isoflavones were not significantly different from that of the air-dried samples ($p > 0.05$), where temperature-induced degradation is unlikely. Alternatively, perhaps extraction was slower in oven- or air-dried material with larger glycosylated molecules.

As can be seen from the ratios in **Figure 3**, the percentage of aglycones in oven-dried material was relatively low at 9%. Some β-glucosidase-mediated hydrolysis of the glucosides to their aglycone equivalents could have occurred after harvest in oven-dried material because this enzyme is still stable at elevated temperatures (*25*); however, it is apparent that this was relatively limited. Presumably, hydrolysis was restricted because the oven-drying process of 3 h was relatively rapid.

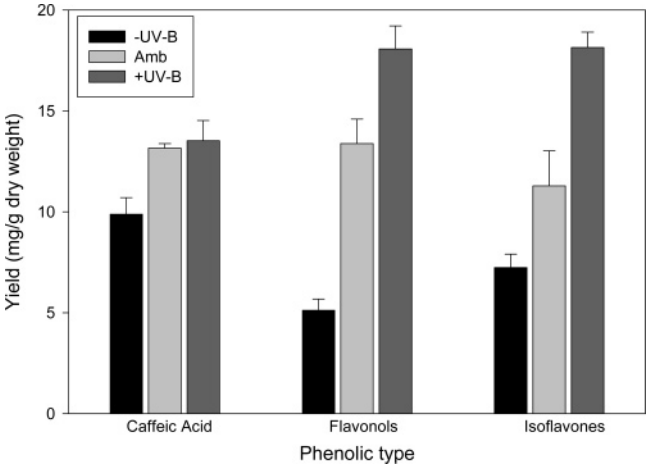


Figure 5. Effect of the UV-B radiation on the yield of total caffeic acid, flavonols, and isoflavones (formononetin and biochanin-A, expressed as aglycone equivalents) in vacuum-dried samples. The −UV-B treatment was 5% of the ambient treatment, and the +UV-B was maintained at 25% higher than ambient. Data shown are mean ($n = 7$ for all data points) and standard error.

The vacuum-dried plant material was in a hydrated state for up to 2 days, during which time extensive enzymatic hydrolysis could have occurred, resulting in almost complete conversion of the isoflavone glycosides to aglycones. In the late season air-dry harvest, which was performed 6 weeks later than this, the percentage of aglycones was approximately half that for vacuum drying (41 versus 95%). The first air-dried harvest had lower levels of aglycones (17%), and the oven-dried leaves had an even lower percentage (9%) (**Figure 3**). Each of these percentages were significantly different from each other ($p < 0.01$). This gradient of aglycone concentration reflects the length of the drying period and consequently the time available for enzymatic hydrolysis. The conversion of glucosylated isoflavones to their aglycones may have been further enhanced in the vacuum-dried samples because the freezing and thawing process would have ruptured cell membranes, releasing and mixing the compounds with the enzyme.

These studies demonstrate that the highest yields of isoflavones will be obtained using the vacuum-drying method. In addition, under this method, 95% of these compounds will be converted to their biologically active aglycone conjugates. On a commercial scale, however, this method is probably too expensive. Air drying affords a cheaper compromise, although both the yield and the proportion of aglycones is lower.

UV-B Effect on Isoflavone Yield. Leaves from plants grown under enhanced UV-B (+UV-B), ambient UV-B, and very low UV-B (−UV-B) were harvested and dried by the vacuum-drying method to afford maximum conversion to the aglycones. While plants exposed to enhanced UV-B displayed less vigorous plant growth, HPLC analysis showed that these plants contained markedly higher total formononetin and biochanin A isoflavone levels (ca. 150%) than those exposed to ambient levels of UV-B. These increases were highly significant ($p < 0.01$). The results are summarized in **Figure 5**. This increase in the isoflavone level with enhanced UV-B radiation parallels that observed for other flavonoids in various studies (*13*−*17*, *21*). It is now well-established that UV-B radiation and other stressors cause an upregulation of many of the genes in the phenylpropanoid pathway. Because of this environmentally induced upregulation, the yields from the vacuum-, air-, and oven-dried material described above were higher than for the freeze-dried material collected later in the summer when ambient UV-B levels were lower (UV-B data not shown). It should also be noted that the plants were germinated in the absence of UV-B radiation. Placement of the seedlings in ambient and elevated UV-B may have triggered an overestimation of the UV effect, which might not be entirely comparable to plants grown outside in the presence of UV-B during germination. On the basis of the findings in the present study, we recommend that commercial harvesting of red clover for maximum isoflavone yield should be after a period of fine weather as close to midsummer as is practical, when ambient levels of UV-B are at their maximum. However, it should be noted that other factors such as maturity, temperature, soil moisture availability, etc. might also affect isoflavone yield (*26*, *27*).

Flavonols and Cinnamic Acids. HPLC analysis (**Figure 2**) of the red clover leaves showed that the predominant cinnamic acid was caffeic acid (peak 1), while the flavonols were all derivatives of quercetin (peaks 2−4). The trends with the drying method observed with the isoflavones were duplicated with these compounds also and reinforce the conclusion that the method of drying of samples will influence flavonoid yield. In addition, the UV-B trend found with isoflavones was repeated with flavonols and caffeic acid. Analysis of the effect of UV-B treatment in these compounds showed that the increase in caffeic acid for +UV-B was relatively small (ca. 40%) but was large for the flavonols (ca. 250%) as shown in **Figure 5**. These observations are consistent with previous observations (*13*−*17*) and illustrate that maximum yields for flavonoid compounds will be obtained following a period of UV-B radiation.

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LITERATURE CITED

(1) Wiseman, H. The therapeutic potential of phytoestrogens. *Exp. Opin. Invest. Drugs* **2000**, *9*, 1829−1840.

(2) Peterson, G.; Barnes, S. Gensitein inhibition of the growth of human breast cancer cells: Independence from estrogen receptors and the multi-drug resistance gene. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 661−667.

(3) Peterson, G.; Barnes, S. Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* **1993**, *22*, 335−345.

(4) Carroll, K. K.; Kurowska, E. M. Soy consumption and cholesterol reduction: A review of animal and human studies. *J. Nutr.* **1995**, *125*, 594S−597S.

(5) Liu, J.; Burdette, J. E.; Xu, H.; Gu, C.; van Breeman, R. B.; Bhat, K. P. L.; Booth, N.; Constantinou, A. I.; Pezzuto, J. M.; Fong, H. H. S.; Farnsworth, N. R.; Bolton, J. L. Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. *J. Agric. Food Chem.* **2001**, *49*, 2472−2479.

(6) Setchell, K. D. R.; Brown, N. M.; Desai, P.; Zimmer-Nechemias, L.; Wolfe, B. E.; Brashear, W. T.; Kirsheer, A. S.; Cassidy, A.; Heubi, J. E. Bioavailabilty and analysis of commercial soy isoflavone supplements. *J. Nutr.* **2001**, *131*, 1362S−1375S.

(7) Setchell, K. D. R.; Cole, S. J. Variations in isoflavone levels in soy foods and soy protein isolates and issues related to isoflavone databases and food labelling. *J. Agric. Food Chem.* **2003**, *51*, 4146−4155.

(8) Prasain, J. K.; Jones, K.; Kirk, M.; Wilson, L.; Smith-Johnson, M.; Weaver, C.; Barnes, S. Profiling and quantification of isoflavonoids in kudzu dietary supplements by high performance liquid chromatography and electrospray ionisation tandem mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 4213−4218.
(9) Swinny, E. E.; Markham, K. R. Applications of flavonoid analysis and identification techniques. In *Flavonoids in Health and Disease*, 2nd ed.; Rice-Evans, C. A., Packer, L. Eds.; Marcel Dekker: New York, 2003; chapter 4, pp 97−122.
(10) de Rijke, E.; Zafia-Gomez, A.; Ariese, F.; Brinkman, U. A.; Gooijer, C. Determination of isoflavone glucoside malonates in T*rifolium pratense* L. (red clover) extracts: Quantification and stability studies. *J. Chromatogr., A* **2001**, *932*, 55−64.
(11) Lin, L.; He, X.; Lindenmaier, M.; Yang, J.; Qiu, S.; Cordell, G. A. LC−ESI−MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*). *J. Agric. Food Chem.* **2000**, *48*, 354−365.
(12) He, X.; Lin, L.; Lian, L. Analysis of flavonoids from red clover by liquid chromatography electrospray mass spectrometry. *J. Chromatogr., A* **1996**, *755*, 127−132.
(13) Ryan, K. G.; Swinny, E. E.; Markham, K. R.; Winefield, C. Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves. *Phytochemistry* **2002**, *59*, 23−32.
(14) Ryan, K. G.; Swinny, E. E.; Winefield, C.; Markham, K. R. Flavonoids and UV photoprotection in *Arabidopsis* mutants. *Z. Naturforsch., C: J. Biosci.* **2001**, *56*, 745−754.
(15) Jordan, B. R. The effects of ultraviolet-B radiation on plants: A molecular perspective. *Adv. Bot. Res.* **1996**, *22*, 98−162.
(16) Hofman, R. W.; Swinny, E. E.; Bloor, S. J.; Markham, K. R.; Ryan, K. G.; Campbell, B. D.; Jordan, B. R.; Fountain, D. W. Responses of nine white clover populations to ultraviolet-B radiation: Differential flavonoid glycoside accumulation and linkages to biomass production. *Ann. Bot.* **2000**, *826*, 527−537.
(17) Hofman, R. W.; Campbell, B. D.; Bloor, S. J.; Swinny, E. E.; Markham, K. R.; Fountain, D. W. Responses to UV-B radiation in *Trifolium repens* L.—Physiological links to plant productivity and water availability. *Plant Cell Environ.* **2003**, *26*, 603−612.
(18) Walsh, K. R.; Zhang, C. Y.; Vodovitz, Y.; Schwartz, S. J.; Failla, M. J. Stability and bioaccessibility of isoflavones from soy bread during *in vitro* digestion. *J. Agric. Food Chem.* **2003**, *51*, 4603−4609.
(19) Setchell, K. D. R.; Brown, N. B.; Zimmer-Nechemias, L.; Brasheer, W. T.; Wolfe, B.; Kirsher, A. S.; Heubi, J. Evidence for lack of absorption of soy isoflavone glycoside in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am. J. Clin. Nutr.* **2002**, *76*, 447−452.
(20) Summer, L. W.; Paiva, N.; Dixon, R. A.; Geno, P. W. High performance liquid chromatography/continuous-flow liquid secondary ion mass spectrometry of flavonoid glycosides in leguminous plant extracts. *J. Mass Spectrom.* **1996**, *31*, 472−485.
(21) Ryan, K. G.; Markham, K. R.; Bloor, S. J.; Bradley, J. M.; Mitchell, K. A.; Jordan, B. R. UV-B radiation induced increase in quercetin:kaempferol ratio in normal and transgenic lines of *Petunia. Photochem. Photobiol.* **1998**, *68*, 323−330.
(22) Ryan, K. G.; Ireland, W. A small indoor plant growth chamber with modulated enhancement of solar UV-B radiation. *J. Environ. Qual.* **1997**, *26*, 866−871.
(23) Ryan, K. G.; Smith, G. J.; Rhoades, D. A.; Coppell, B. B. Erythermal ultraviolet insolation in New Zealand at solar zenith angles of 30° and 45°. *Photochem. Photobiol.* **1996**, *63*, 628−632.
(24) Toebes, A H. W.; de Boer, V.; Verkleij, J. A. C.; Lingeman, H.; Ernst, W. H. O. Extraction of isoflavone malonylglucosides from *Trifolium pratense* L. *J. Agric. Food Chem.* **2005**, *53*, 4660−4666.
(25) Hsieh, M.-C.; Graham, T. L. Partial purification and characterization of a soybean β-glucosidase with high specific activity towards isoflavone conjugates. *Phytochemistry* **2001**, *55*, 481−504.
(26) Hofmann, R. W.; Campbell, B. D.; Bloor, S. J.; Swinny, E. E.; Markham, K. R.; Ryan, K. G.; Fountain, D. W. Responses to UV-B radiation in *Trifolium repens* L.—Physiological links to plant productivity and water availability. *Plant Cell Environ.* **2003**, *26*, 603−612.
(27) 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.

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