JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Extraction of Isoflavone Malonylglucosides from *Trifolium pratense* L.

ANTOINETTE H. W. TOEBES,*,† VINCENT DE BOER,† JOS A. C. VERKLEIJ,† HENK LINGEMAN,‡ AND WILFRIED H. O. ERNST†

Department of Ecology and Physiology of Plants, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands, and Department of Analytical Chemistry and Applied Spectroscopy, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

Extraction of isoflavone malonylglucosides from red clover (*Trifolium pratense* L.) is a complicated procedure. This is due to the relatively unstable character of the thermolabile glucoside malonates as well as by action of native β-glucosidases, resulting in a rapid degradation of malonylated glucosides into their corresponding aglucones. In this study, Tris was identified as a suitable β-glucosidase inhibitor in red clover extracts, optimized at 350 mM Tris in 80% ethanol at pH 7.2. Extraction of fresh red clover leaves using Tris increased the concentration of malonate conjugated isoflavones approximately 13 to 24 times as opposed to extraction without Tris. A comparison of isoflavone profiles obtained after extraction with and without Tris of different plant organs of red clover and several species within the family Fabaceae suggests that the amount and/or activity of the degenerative β-glucosidase enzymes vary for the different plant parts of red clover and among the species studied. Therefore, the use of standard extraction methods may well result in overestimation of the concentration of aglucones and consequently underestimation of the malonylglucoside isoflavones concentration depending on the plant species and plant part studied.

KEYWORDS: ***Trifolium pratense*; Fabaceae; red clover; extraction method; glucoside malonates; isoflavones**

INTRODUCTION

The isoflavonoids are biphenolic compounds mainly found in members of the legume family Fabaceae. These relative high hydrophobic compounds are not only toxic to different organisms such as fungi and other pathogens but also to the synthesizing plant itself (*1*). To avoid autotoxicity, isoflavones are stored in the vacuole predominantly as the more polar malonylglucoside conjugates (*2*). Deconjugation of isoflavone malonylglucosides is catalyzed by two enzymes, isoflavone malonylglucoside malonylesterase (IEST) and isoflavone glucoside glucosidase (IGLC), as described for chickpea (*3*, *4*) (Figure 1). IEST is an enzyme bound to the tonoplast membrane of the vacuole, whereas IGLC, a β-glucosidase, is supposed to be a cytosolic protein (*5*) possibly localized in the apoplasm (*6*). In contrast, in soybean only one enzyme seems to catalyze the degradation of glucosides. A partially purified glucosidase was able to hydrolyze both the glucosyl and malonyl glucosyl isoflavones directly with similar kinetics seemingly uninhibited by the presence of malonyl. No evidence for an esterase activity was found in the metabolic pathway (*6*).

The separation of isoflavone malonylglucosides and degradative enzymes represent an important regulatory system for the generation of aglucones, which can act directly or intermediary as stimulatory factors activating the nod genes of *Rhizobium* bacteria as well as inhibitory factors against pathogen attack and disease resistance (*7*). During the extraction of isoflavones, the compartmentation of the cell is disrupted whereupon isoflavone glucosides can be readily converted into their corresponding aglucones due to the native β-glucosidase activity in extracts. For that reason, an adequate sample pretreatment is essential in order to determine the correct conjugation of the isoflavones in plant tissue. The objective of this study is to find an appropriate inhibitor of plant β-glucosidase in order to obtain an easy extraction method suitable for the detection of isoflavones in the correct configuration as found *in planta*. Furthermore, variation in degradation of conjugated isoflavones during the extraction procedure was studied in different plant organs of various cultivars of *Trifolium pratense* L. and for several species of Fabaceae.

MATERIALS AND METHODS

Reagents and Solvents. All isoflavone standards were purchased from Roth (Karlsruhe, Germany) except biochanin A, daidzein (Sigma, Zwijndrecht, The Netherlands), and sissotrin (Extrasynthese, Geray, France). The internal standard flavon was acquired from Sigma (Zwijndrecht, The Netherlands).

* Corresponding author. Fax: +31-20-5987123. E-mail: atoebes@bio.vu.nl.
† Department of Ecology and Physiology of Plants.
‡ Department of Analytical Chemistry and Applied Spectroscopy.

10.1021/jf047995f CCC: $30.25
Published on Web 05/17/2005

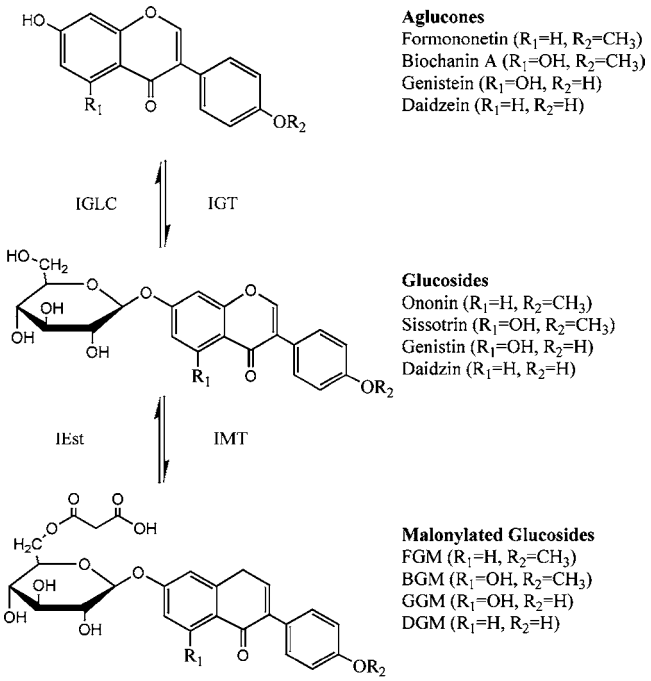

Figure 1. Structures of the isoflavones studied. Glucosides: ononin, formononetin 7-*O*-glucoside; sissotrin, biochanin A 7-*O*-glucoside; genistin, genistein 7-*O*-glucoside; daidzin, daidzein 7-*O*-glucoside. Malonylated glucosides: FGM, formononetin 7-*O*-glucoside-6″-*O*-malonate; BGM, biochanin A 7-*O*-glucoside-6″-*O*-malonate; GGM, genistein 7-*O*-glucoside-6″-*O*-malonate; DGM, daidzein 7-*O*-glucoside-6″-*O*-malonate. Enzymes: IGLC, isoflavone glucoside glucosidase; IEST, isoflavone malonylglucoside malonylesterase; IMT, isoflavone glucoside malonyltransferase; IGT, isoflavone 7-O-glycosyltransferase.

Plant Material and Germination. Red clover seeds (*Trifolium pratense* L.) cv. Essex red were obtained from the Henry Doubleday Research Association, Coventry, U.K., and cultivars Formica, Milvus, Temara, and Vanessa were obtained from the Eidgenössische Forschungsanstalt für landwirtschaftlichen Pflanzenbau, Reckenholz, Switzerland. The seeds were germinated at room temperature (20 ± 1 °C) for approximately 14 days in Petri dishes on paper tissues covered with a paper filter. Subsequently, the seedlings were put on commercial potting soil (Jongkind BV, Aalsmeer, The Netherlands) and grown in a controlled greenhouse compartment (illumination with mercury high vapor lamps for a 14 h period; temperature, 20 ± 4 °C; relative humidity (RH) 70 ± 10%). The other species belonging to the Fabaceae were *Genista anglica* L., *Trifolium hybridum* L., and *Trifolium repens* L. They were collected from several locations in The Netherlands and grown under identical greenhouse conditions for over 6 months in order to adjust them to identical soil and environmental conditions. The plants were rotated frequently within the greenhouse compartment in order to minimize effects caused by variation in light intensities. After the adjustment period in the controlled greenhouse compartment leaves, flowers and stems were collected for analysis. Only those plant parts were collected that were newly formed under greenhouse conditions from adult plants which had flowered at least once.

Extraction Procedure. Fresh plant material was homogenized in liquid nitrogen using mortar and pestle. From the powdery homogenate 0.5 g was used for the extraction of isoflavones using mortar, pestle, quartz sand, 6 mL of extraction solvent, and 50 mM of the internal standard flavon at room temperature (20 ± 1 °C). Extraction solvents used were 80% ethanol, 80% acetonitrile, or 80% acetone. Degradation of isoflavones conjugates into their corresponding aglucones was studied at various concentrations of Tris dissolved in 80% EtOH and at several pH values. The Soxhlet method is an extraction method with high yield due to the constant supply of fresh solvent. Prior to placing plant material in the Soxhlet, the homogenized material was boiled in 20−30 mL of ethanol for 10 min to inhibit enzyme activity. After extraction, the homogenate was centrifuged for 5 min in an Eppendorf centrifuge (Microcentaur, MSE, GB) at 13000 rpm at room temperature (20 ± 1 °C). The supernatant was filtered over a 0.22 μm Costar Spin-X centrifuge filter (Costar Corporation, Cambridge, MA) prior to injection.

HPLC Analysis of Isoflavones. Extracted isoflavones (injection volume 6 μL) were separated on a reversed-phase Novo-Pak C_{18} analytical column (60 Å, 4 μm, 3.9 × 150 mm, Waters, Millford) at 37 °C using a gradient of acetonitrile and water, both containing 0.1% (v/v) TFA at a flow rate of 0.6 mL min^{-1}. Prior to injection, the column was equilibrated in 15% (v/v) acetonitrile and 85% (v/v) water. The gradient used, with a total analysis time of 62 min, started isocratic at 15% (v/v) acetonitrile for 15 min followed by a linear gradient up to 39% (v/v) acetonitrile in 20 min and finally a linear gradient to 64% (v/v) acetonitrile for 5 min. The column was cleaned with 95% (v/v) acetonitrile for 10 min and reequilibrated in 15% (v/v) acetonitrile for 12 min. A Waters 996 photodiode array detector monitored absorbance from 240 to 420 nm used for identification of the individual isoflavones. Identification of daidzein, genistein, formononetin and biochanin A, ononin, and sissotrin was performed using standard compounds. Those isoflavones which were not commercially available were identified by the method of De Rijke (*8*) using LC−MS. Quantification was performed by integrating the analytical data at 254 nm using the Waters Millennium chromatography management software.

RESULTS AND DISCUSSION

Isoflavone Malonylglucoside Extraction. Many studies focus on maximizing isoflavone recovery from plant material. The focal point has been directed toward the accurate quantification of isoflavones in plants often by hydrolyzing all conjugated isoflavones into aglucones. However, hardly any attention is paid to an appropriate sample pretreatment in order to get a realistic reflection of the configuration of isoflavones as found *in planta*. Studies analyzing isoflavone concentrations in red clover (*Trifolium pratense*) by HPLC often report high quantities of aglucones and glucosides, and consequently, a low concentration of malonylated glucosides indicating deconjugation of isoflavones (Table 1). Isoflavone malonylglucoside extraction from fresh red clover leaves is hampered by the relative unstable character of the conjugated glucosidic isoflavones and the presence of native β-glucosidase enzymes in extracts. The extent of isoflavone degradation was unaffected by the extraction solvent used since fresh leaf extraction with acetonitrile, ethanol or acetone at room temperature (20 ± 1 °C) resulted primarily in unsubstituted isoflavones (Table 2). Neither was the degradation of conjugated isoflavones affected by the concentration of the apolar extraction solvent used, given that ethanol extraction (-Tris) at concentrations ranging from 0% to 90% ethanol predominantly resulted in aglucones (Table 3). This absence of the more hydrophilic isoflavone (malonyl)-glucosides was not the result of hindered solubility caused by the polarity of the extraction solvent used as these compounds were missing in the entire range of ethanol concentrations used (Table 3). Presumably, β-glucosidase activity was unaffected by these factors resulting in a substantial degradation of conjugated isoflavones. Preheating the extract in order to inactivate the hydrolyzing enzymes was inadequate since β-glucosidase is highly heat stable and remains substantially active (87%) after 45 min at 50 °C (*6*). Heating also caused a loss of malonate conjugated isoflavones as seen using the Soxhlet method which gave 26% malonylated glucosides and 70% glucosides. Although this method usually results in a higher yield, in this study the extensive boiling of the extraction solvent converted nearly all malonylated glucosides. Extraction at 0 °C gave high quantities of isoflavone malonylglucosides in comparison to extraction at room temperature ($p < 0.001$), however, the slightest temperature rise may well result in an increase in

Table 1. Percentage of Isoflavone Malonate Glucosides (MG), Isoflavone Glucosides (G), and Aglucons (A) of Total Isoflavone Concentrations in Red Clover Analyzed by HPLC[a]

ref	plant material	analysis	isoflavones		
			% MG	% G	% A
Franke et al. 1994 (*21*)	red clover sprouts (freeze-dried)	96% EtOH + 10 M HCl	–	–	100[b]
Vetter 1995 (*17*)	red clover (dried)	MeOH (55 °C, shaken)	–	–	100
He et al. 1996 (*18*)	red clover flowers (dried)	70% EtOH (refluxed), MeOH (1:5)	–	69[b]	31[b]
Edwards et al. 1997 (*13*)	red clover seedlings foliage	ice-cold acetone (grinded), acetone, acetone/MeOH (1:1 v/v), evaporated, 1 M HCl (pH 2), ethyl acetate, dried, MeOH + acidified H_2O (1:4 v/v)	95	–	5
Lin et al. 2000 (*15*)	red clover (dried)	MeOH/H_2O (9:1 v/v; sonification)	77[b]	17[b]	6[b]
Wu et al. 2003 (*16*)	red clover (air-dried)	80% EtOH + HCl (7:8 v/v; refluxed), evaporated, aqueous ammonia until pH 6, EtOAc, 10–50% MeOH (solid-phase extraction), 10% MeCN (prep-HPLC)	58[b]	17[b]	25[b]

[a] Analysis extraction solution: ethanol (EtOH), methanol (MeOH), ethyl acetate (EtOAc), acetonitrile (MeCN). – = not detected. [b] Percentages determined by peak height.

Table 2. Effect of Isoflavone Extraction Using a Variety of Solvents at Different Temperatures[a]

extraction solvent	*T* (°C)	% isoflavones[b]			total isoflavones (μmol g FW^{-1})
		MG	G	A	
acetone	20	12	9	79	53
acetonitrile	20	9	5	86	53
ethanol	20	9	6	85	53
ethanol	0	75	5	20	51
EtOH + 350 mM Tris	20	88	6	6	53

[a] Fresh red clover (*Trifolium pratense;* cv. Essex red) leaves were ground with either 80% ethanol (EtOH), 80% acetonitrile, 80% acetone, or 350 mM Tris in 80% ethanol pH 7.2 at 0 or 20 °C. Values given are relative to the total concentration of isoflavones (% of total isoflavone concentration). The samples were taken from a homogeneous batch of fresh red clover leaves homogenized with liquid nitrogen to avoid differences caused by intraspecific variation. [b] MG = isoflavone malonate glucosides, G = isoflavone glucosides, A = aglucones.

Table 3. Efficiency of Extraction of Isoflavones (μmol g/FW) from Fresh Red Clover Leaves (*Trifolium pratense;* cv. Essex Red) Using Various Concentrations of EtOH with or without 350 mM Tris (pH 7.2)[a]

EtOH (%)	isoflavones (μmol g FW^{-1})[b]							
	– Tris				+ Tris			
	MG	G	A	total	MG	G	A	total
0	–	–	1	1	2	3	4	9
10	–	–	1	1	6	3	3	12
20	–	–	1	1	14	4	3	20
30	–	–	5	5	19	3	3	25
40	–	–	7	7	37	4	11	52
50	1	–	13	14	37	3	11	50
60	2	2	37	40	39	3	10	52
70	3	2	48	53	42	3	7	51
80	3	3	47	53	44	3	5	52
90	4	3	47	54	41	3	5	49

[a] The samples were taken from a homogeneous batch of fresh red clover leaves homogenized with liquid nitrogen to avoid differences caused by intraspecific variation. [b] MG = isoflavone malonate glucosides, G = isoflavone glucosides, A = aglucones, – = not detectable.

aglucones (Table 2). Therefore, inhibition of β-glucosidases is desirable and can be accomplished by a wide variety of substances. Dale et al. (*9*) reported that various amines inhibited these enzymes reversibly. Glycosamine was recognized as the best inhibitor by Legler (*10*), but this compound needs to be synthesized and includes a difficult crystallization process.

Table 4. Effect of Extraction of Fresh Red Clover (*Trifolium pratense)* Leaves Using Various Concentrations of Tris in 80% EtOH (pH 7.2) (A) or 350 mM Tris in 80% EtOH at Various pH Values (B) on the Relative Amount of the Various Isoflavone Configurations[a,b]

			% isofavones using Tris (mM)							
			0	1	10	50	125	250	350	500
A	MG	FGM	29	32	41	48	50	50	50	50
		BGM	19	22	29	31	37	37	37	37
	G	FG	6	6	6	6	6	6	6	6
		BG	1	1	2	2	2	2	2	2
	A	F	20	17	10	6	3	3	3	3
		B	25	22	12	7	2	2	2	2
		total (μmol g FW^{-1})	26	27	32	35	36	36	37	37

			% isoflavones at various pH							
			2	4	5	7	7.2	7.4	8	9
B	MG	FGM	49	48	49	49	49	48	43	37
		BGM	35	35	35	35	35	35	31	25
	G	FG	6	6	6	8	8	9	14	21
		BG	2	2	2	3	3	4	8	13
	A	F	4	4	4	3	3	2	2	2
		B	4	5	4	2	2	2	2	2
		total (μmol g FW^{-1})	27	27	27	27	35	30	28	25

[a] Values (%) given are relative to the total concentration of isoflavones (μmol g FW^{-1}). The samples were taken from a homogeneous batch of fresh red clover leaves homogenized with liquid nitrogen to avoid differences caused by intraspecific variation. [b] Malonylated glucosides (MG): FGM, formononetin 7-*O*-glucoside-6″-*O*-malonate; BGM, biochanin A 7-*O*-glucoside-6′-*O*-malonate. Glucosides (G): FG, formononetin 7-O-glucoside; BG, biochanin A 7-O-glucoside. Aglucones (A): F, formononetin; B, biochanin A. –, not detectable.

Larner and Gillespie (*11*) investigated the starch digestion in the small intestine and reported that following α-amylase action the hydrolysis of saccharides is completed by α-glucosidase. They identified several inhibitors, such as histidine and Tris. It was established by Hsieh and Graham (*6*) that silver and mercury ions were the best inhibitors, however the inhibitors discussed previously were not studied. From this variety of β-glucosidase inhibitors Tris was selected as a possible candidate for the inhibition of plant β-glucosidase for the reason that Tris is readily available. As shown in Table 4A the concentration of 350 mM Tris (pH 7.2) was optimal for extraction of the isoflavone malonylglucosides in red clover. Concentrations lower than 125 mM Tris resulted in a substantial increase of aglucones and consequently a decrease in (malonyl)glucosides

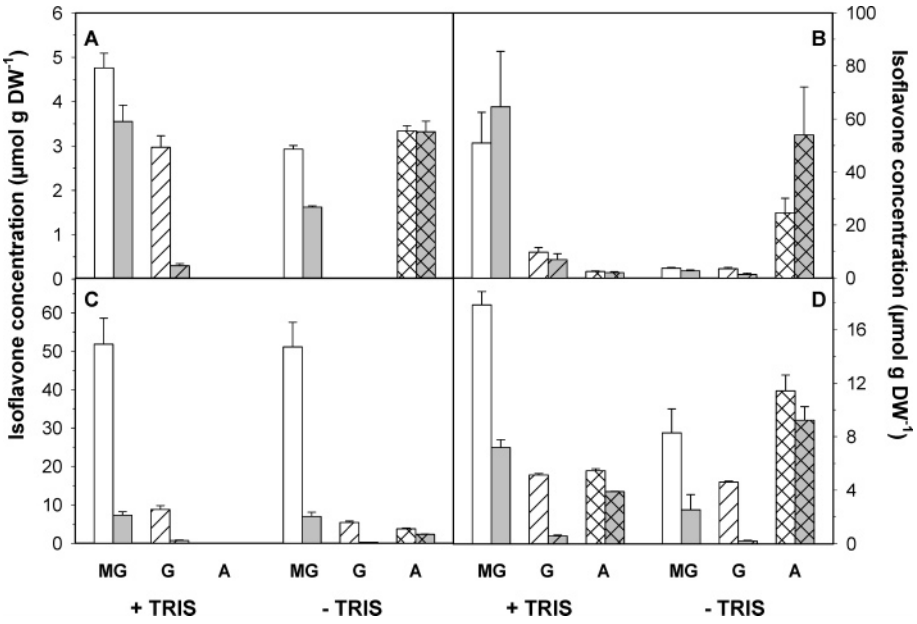

Figure 2. Concentration of isoflavones in flowers (A), leaves (B), stem (C), and roots (D) of *Trifolium pratense* (cv. Formica) after extraction using 80% EtOH with (+Tris) or without (–Tris) 350 mM Tris (pH 7.2). Malonylated glucosides (MG): FGM (white bars), BGM (gray bars). Glucosides (G): ononin (white single patterned bars), sissotrin (gray single patterned bars). Aglucones (A): formononetin (white double patterned bars) and biochanin A (gray double patterned bars). Values given are means ± SEM of three replications, each taken from individual plants.

and concentrations below 350 mM Tris affected the total isoflavone concentrations. The conjugation of isoflavones was also affected by the pH with an optimal yield at pH 7.2 using a concentration of 350 mM Tris (Table 4B). Alkaline extraction resulted in separation of the malonyl group producing 7-*O*-glucosides. In contrast, acidic extraction produced more aglucones parallel with a decline in isoflavone glucosides. It can be concluded that grinding fresh red clover leaves using 350 mM Tris in 80% EtOH at pH 7.2 is a quick and easy extraction procedure resulting predominantly in malonylated isoflavone glucosides. The 88% of malonylated glucosides measured in this study **(**Table 2**)** exceed those reported in most studies (Table 1) as confirmed by De Rijke et al. (*12*). With our collaboration, De Rijke et al. (*12*) used the Tris extraction developed and optimized in this report and stated that the glucoside-malonate peaks measured in their study were higher than those observed in most studies due to the protective action of Tris. Edwards et al. (*13*) detected the highest percentage of malonylated glucosides. However, this study did not detect any demalonylated derivatives of FGM and the position of the detected biochanin A-7-*O*-glucoside peak in the chromatogram does not match the position reported for sissotrin by the other studies. Therefore, the lack of isoflavone glucosides may cause the percentage of aglucones to be overvalued. The lower quantities of malonylated glucosides reported in most studies indicate that during plant material handling and the extraction procedure malonate and sugar groups are lost and that addition of Tris to the extraction solvent prohibits or reduces the degradation of (malonylated)-glucosides ($p < 0.001$).

Conjugated Isoflavone Degradation in Various Plant Organs. Given the relative unstable character of the malonylglucosides in red clover leaves, the stability of the conjugated isoflavones in flowers, stem and roots of red clover was studied (Figure 2). In these plant parts, the assumed involvement of the β-glucosidase enzymes seems to be less profound as can be concluded from comparison of isoflavone profiles obtained from extractions with and without Tris. In leaves, the concentration of malonylated glucosides decreased approximately by 90% in the absence of Tris. For flowers, the concentration of FGM and BGM was diminished by, respectively, 38% and 55% (Figure 2a) and in roots by 54% and 65% (Figure 2d). In contrast, Hsieh and Graham (*6*) located the highest specific activity of a soybean isoflavone conjugate-hydrolyzing β-glucosidase in the roots of the seedlings. This discrepancy may well be explained by the difference in developmental stage of the species studied, that is soybean seedlings opposed to adult red clover plants. Moreover, soybean leaves contain only traces of isoflavones (*14*) in contrast to red clover where the highest concentration of isoflavones is located in leaves (Figure 2b). Higher isoflavone concentrations may possibly be correlated to a higher involvement of β-glycosidase enzymes.

The malonated glucosides were the dominant isoflavone form in all plant parts studied. In red clover leaves, BGM was the major isoflavone present as opposed to roots, flowers and stem where FGM was the main compound. Lin et al. (*15*) also found BGM to be the dominant isoflavone in red clover leaves. However, Wu et al. (*16*) reported FGM as the dominant isoflavone in red clover leaves; Vetter (*17*) detected equal concentrations of formononetin and biochanin A related compounds. In flowers, Lin et al. (*15*) and He et al. (*18*) detected glucosides as the dominant isoflavones. In this study, isoflavone glucosides were abundant in flowers, yet the quantities of malonylated glucosides surpassed those found for glucosides. This dissimilarity in isoflavone dominance could be ascribed to intraspecific variation, but differences in growth conditions may also affect isoflavone concentrations. Beside variation in isoflavone composition of the various plant organs studied, the concentration of isoflavones was also variable. In leaves and stems, the total isoflavone concentration surpassed the concentrations measured for flowers and roots. This variation in isoflavone quantities between leaves and flowers was confirmed by Lin et al. (*15*), although Vetter (*17*) detected approximately equal concentrations. Red clover is an important feeding material for sheep and cattle and exhibits estrogenic properties. Since leaves and stems are the parts of the plant most commonly eaten by herbivores, these data could be of importance to evaluate

Table 5. Relative Amounts of Isoflavones (Percent of Total Isoflavone Concentration) in Fresh Leaves, Flowers, Stems, and Roots of Various Red Clover (*Trifolium pratense)* Cultivars Extracted with 80% Ethanol with or without 350 mM Tris (pH7.2)[a]

		% isoflavones[b]					
		+ Tris			− Tris		
	cultivars	MG	G	A	MG	G	A
flower	Formica	72 ± 2.2	28 ± 2.2	–	34 ± 0.7	17 ± 0.4	49 ± 1.0
	Milvus	75 ± 1.4	25 ± 1.4	–	47 ± 1.6	18 ± 0.5	35 ± 1.4
	Vanessa	74 ± 3.2	26 ± 3.2	–	45 ± 0.5	18 ± 1.5	37 ± 0.9
	Temara	72 ± 1.3	28 ± 1.3	–	41 ± 0.9	20 ± 0.6	39 ± 0.6
leaf	Formica	84 ± 0.9	12 ± 0.3	4 ± 0.6	8 ± 2.4	6 ± 0.5	86± 2.7
	Milvus	86 ± 1.2	11 ± 0.6	3 ± 0.6	9 ± 0.7	7 ± 0.3	84 ± 0.9
	Vanessa	88 ± 0.3	11 ± 0.3	2 ± 0.1	9 ± 2.0	7 ± 0.3	84 ± 2.1
	Temara	85 ± 0.1	12 ± 0.2	3 ± 0.3	16 ± 1.3	8 ± 0.1	76 ± 1.2
stem	Formica	86 ± 0.2	14 ± 0.2	–	83 ± 1.9	8 ± 0.5	9 ± 1.5
	Milvus	88 ± 0.3	12 ± 0.3	–	85 ± 1.1	8 ± 0.3	7 ± 0.8
	Vanessa	87 ± 0.7	13 ± 0.7	–	87 ± 2.3	7 ± 0.5	6 ± 1.9
	Temara	87 ± 0.3	13 ± 0.3	–	85 ± 0.9	8 ± 0.4	7 ± 1.2
root	Formica	62 ± 1.5	14 ± 0.5	23 ± 1.0	29 ± 6.7	14 ± 1.0	57 ± 6.1
	Milvus	62 ± 1.8	12 ± 0.5	25 ± 1.4	43 ± 7.7	12 ± 0.8	46 ± 6.9
	Vanessa	71 ± 4.6	13 ± 0.4	17 ± 4.4	43 ± 6.2	12 ± 1.3	45 ± 5.9
	Temara	56 ± 4.5	16 ± 1.1	29 ± 3.4	29 ± 2.9	16 ± 1.5	55 ± 1.4

[a] Values given are means ± SEM of three replications, each taken from individual plants. [b] MG = isoflavone malonate glucosides, G = isoflavone glucosides, A = aglucones, – = not detectable.

the potential toxicological impact on herbivores. Although the concentration of biochanin A related compounds in stems are approximately nine times lower than those found for leaves, the endocrine disrupting effects for stems and leaves are expected to be similar for the reason that total formononetin was found in similar concentrations and the estrogenicity of pastures for grazing sheep is thought to be related to the level of formononetin and its derivatives (*19*). In stems, no significant reduction of malonylated glucosides was observed (Figure 2c), suggesting the absence or inactivity of isoflavone malonylglucoside degradative enzymes.

The absolute concentrations of isoflavones showed considerable variation for individual red clover plants (Figure 2), although the distribution of the different isoflavone forms relative to the total amount of isoflavones was less variable (Table 5). A comparable pattern in distribution of the various isoflavone forms and altering isoflavone profile after extraction with and without Tris was observed for three other cultivars of red clover (Table 5). This observation strengthens the previous conclusion of a variable involvement of β-glucosidase in the different plant parts studied. Hsieh and Graham (*6*) found no evidence for a separate malonylesterase activity in any soy tissue, yet a partially purified glucosidase was able to hydrolyze both the glucosyl and malonyl glucosyl isoflavones directly with similar kinetics seemingly uninhibited by the presence of malonyl. In contrast, almond emulsion β-glucosidase is unable to hydrolyze malonylglucosyl isoflavone conjugates and in chickpea the malonyl and glucose are removed respectively by a malonylesterase and β-glucosidase (*2*−*4*). The observation that both malonylated glucosides and to a lesser extent glucosides were converted into aglucones in red clover does not indicate that malonylesterase is involved since both compounds could serve as a substrate for β-glucosidase as described for soy (*6*). However, in both chickpea and red clover, the main isoflavones are the 4′-*O*-methylated isoflavones formononetin and biochanin A, suggesting that possibly both malonylesterase and β-glucosidase are active in red clover as described for chickpea. The isoflavone profiles of soy and red clover are less comparable for the reason that for soy only genistein and daidzein metabolites are reported. Therefore, one might hypothesize that the option of β-glucosidase using both malonylated glucosides and glucosides as a substrate is less likely. Especially since chickpea isoflavone-specific β-glucosidase has a higher specificity toward formononetin and biochanin A glucosides than the soy isoflavone specific β-glucosidase (*6*). The hypothesis of an esterase involvement is strengthened by the observation that following inhibition of β-glucosidase activity in red clover flowers higher quantities of isoflavone glucosides were detected. As to why this rise in isoflavone glucosides was not observed for the other plant organs is unclear. The absence or inactivity of the malonylesterase in these plant organs is an option, however, since almond β-glucosidase is unable to hydrolyze malonylglucosyl isoflavone conjugates the observed deconjugation of malonylated isoflavones resulting primarily in the formation of aglucones in red clover after extraction without Tris is unexpected. Therefore, purification and characterization of the isoflavone deconjugating enzymes in red clover is warranted to determine whether β-glucosidase is solely involved in the modification of isoflavones or malonylesterase as well.

Conjugated Isoflavone Degradation in Various Fabaceae Species. The prominent involvement of β-glucosidase in the deconjugation of isoflavone glucosides as seen in the leaves of red clover provoked questions about the involvement of β-glucosidase in leaves of several other members of the Fabaceae plant family. Isoflavone extraction from fresh leaves of several Trifolium species, grown under identical conditions, showed that the isoflavone concentration and the extent to which isoflavones were deconjugated differed considerable among the species studied (Table 6). From *Trifolium repens* (white clover), only FGM and formononetin were extracted which is consonant with the results reported by Wu et al. (*16*) who also detected mainly formononetin related isoflavones in white clover leaves. Inhibition of β-glucosidase activity with Tris suppressed the formation of the aglucone formononetin from FGM but also the degradation of other formononetin conjugates since the increase of the aglucone surpasses the decline of FGM after extraction without Tris. The main isoflavones of *Trifolium hybridum*, FGM and GGM, were hardly reduced without β-glucosidase inhibition. Genistein, the corresponding aglucone of GGM, barely increased; however, the formononetin concentration did rise above the detection limit which again could be

Table 6. Isoflavone Concentration in Several Plants Belonging to the Family Fabaceae. Fresh leaves were extracted with 80% ethanol with or without 350 mM Tris (pH 7.2)

	isoflavones (μmol g DW^{-1})								
	GGM	GG	G	FGM	FG	F	BGM	BG	B
+ Tris									
Trifolium pratense	–	–	–	50.9 (11.6)	9.7 (1.8)	2.5 (0.1)	64.6 (20.9)	7.0 (2.2)	2.1 (0.4)
Trifolium repens	–	–	–	2.9 (0.2)	–	–	–	–	–
Trifolium hybridum	1.5 (0.05)	–	1.0 (0.00)	2.7 (0.3)	–	–	–	–	–
Genista anglica	32.8	15.7	–	–	–	–	–	–	–
– Tris									
Trifolium pratense	–	–	–	3.7 (0.3)	3.5 (0.5)	24.6 (5.5)	2.7 (0.3)	1.4 (0.5)	54.0 (18.1)
Trifolium repens	–	–	–	2.2 (0.2)	–	2.3 (0.05)	–	–	–
Trifolium hybridum	1.1 (0.03)	-	1.1 (0.02)	2.1 (0.2)	–	1.6 (0.04)	–	–	–
Genista anglica	16.2	16.0	9.5	–	–	–	–	–	–

[a] Fresh leaves were extracted with 80% ethanol with or without 350 mM Tris (pH 7.2). Values given are means ± SEM of three replications, each taken from individual plants. [b] Malonylated glucosides: GGM, genistein 7-O-glucoside-6″-O-malonate; FGM, formononetin 7-O-glucoside-6″-O-malonate; BGM, biochanin A 7-O-glucoside-6″-O-malonate. Glucosides: GG, genistein 7-O-glucoside; FG, formononetin 7-O-glucoside; BG, biochanin A 7-O-glucoside. Aglucones: G, genistein; F, formononetin; B, biochanin A. –, not detectable.

the result of the degradation of other formononetin conjugates. High quantities of genistein conjugates were found in *Genista anglica*, and 50% of the malonylated glucoside of genistein was transformed into the corresponding aglucone. These results show that for most of the legume species tested, using standard extraction methods without β-glucosidase inhibition will result in an overestimation of aglucones and consequently an underestimation of the conjugated isoflavones. However, for most species the inaccuracy will be less severe than for red clover for the reason that in red clover extracts nearly all conjugated isoflavones were transformed into there corresponding aglucones using an extraction procedure without Tris buffer.

The instability of malonylglucosyl and glucosyl isoflavone conjugates in extracts of fresh red clover leaves was demonstrated, and Tris was identified as a suitable inhibitor of the degradation of malonylated isoflavone glucosides into their corresponding aglucones. These data suggest a variable involvement of deconjugating enzymes in different plant parts and plant species. Establishing the right isoflavone configuration in plant tissue might not only be relevant from a phytochemical point of view but also of significance in understanding the bioavailability of isoflavones present in human food and important silage for sheep and cattle. The bioavailability of the glucoconjugates might be different from that of the unsubstituted aglucones (*20*). Isoflavones are disease protective components associated with reducing the risk of prostate and breast cancer and chronic diseases such as coronary heart diseases (*21*). Conversely, they are known to cause an infertility syndrome in cattle and sheep (*22*). Food processing or chewing of fodder disrupts the compartmentation of the cell and consequently results in hydrolyzation of the isoflavone conjugates depending on the amount and activity of enzymes converting malonylated isoflavone glucosides into unsubstituted glucosides or aglucones. The resulting isoflavone configuration profiles will vary for different plant species and plants organs as a result of the variable instability of isoflavone conjugates as a consequence of enzyme activity as shown in this study. Thereby the bioavailability of isoflavones in food and fodder may well be affect. This study provides a quick and easy method for measuring the correct configuration of isoflavones.

ACKNOWLEDGMENT

We thank Ms. Eva de Rijke at the Department of Analytical Chemistry and Applied Spectroscopy of the Vrije Universiteit for characterization of the isoflavones, Dr. B. Boller, Eidgenössische Forschungsanstalt für landwirtschaftlichen Pflanzenbau, Reckenholz, Switzerland, for the supply of the red clover seeds, and Paul Koevoets for the technical assistance operating the HPLC and his remarks concerning the manuscript. The critical comments of Dr. Lusi van Heerwaarden on the manuscript are gratefully acknowledged.

Note Added after ASAP Publication: An incorrect species name was listed in Table 6 of the version published ASAP May 17, 2005. The correct version was published ASAP on May 26, 2005.

LITERATURE CITED

(1) Wink, M. Biochemistry, role and biotechnology of secondary metabolites. In *Biochemistry of plant secondary metabolism*; Wink, M., Ed.; Annual Plant Reviews 2; Sheffield Academic Press Ltd.: Sheffield, 1999; pp 1−17.

(2) Mackenbrock, U.; Gunia W.; Barz W. Accumulation and metabolism of medicarpin and maackiain malonylglucosides in elicited chickpea (*Cicer arietinum* L.) cell suspension cultures. *J. Plant Physiol.* **1993**, *142*, 385−391.

(3) Hosel, W.; Barz, W. β-Glucosidases from *Cicer arietinum* L.: purification and properties of isoflavone-7-O-glucoside-specific-β-glucosidases. *Eur. J. Biochem.* **1975**, *57*, 607−616.

(4) Hinderer, W.; Koster, J.; Barz, W. Purification and properties of a specific isoflavone 7-*O*-glucoside-6″-malonate malonylesterase from roots of chickpea (*Cicer arietinum* L.). *Arch. Biochem. Biophys.* **1986**, *148*, 570−578.

(5) Barz, W.; Mackenbrock, U. Constitutive and elicitation induced metabolism of isoflavones and pterocarpans in chickpea (*Cicer arietinum*) cell suspension cultures. *Plant Cell Tissue Organ Cult.* **1994**, *38*, 199−211.

(6) Hsieh, M.-C.; Graham, T. L. Partial purification and characterization of a soybean β-glucosidase with high specific activity towards isoflavone conjugates. *Phytochemistry* **2001**, *58*, 995−1005.

(7) Harborne, J. B. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481−504.

(8) de Rijke, E.; Zafra-Gómez, A.; Ariese, F.; Brinkman, U. A. Th.; Gooijer, C. Determination of isoflavone glucoside malonates in*Trifolium pratense* L. (red clover) extracts: quantification and stability studies. *J. Chromatogr. A* **2001**, *932*, 55−64.

(9) Dale, M. P.; Ensley, H. E.; Kern, K.; Sastry, A. R.; Byers, L. D. Reversible inhibitors of β-glucosidase. *Biochemistry* **1985**, *24*, 3530−3539.

(10) Legler, G. Inhibition of β-glucosidases from almonds by cationic and neutral β-glycosyl derivates. *Biochim. Biophys. Acta* **1978**, *524*, 94−101.

(11) Larner, J.; Gillespie, R. J. Gastrointestinal digestion of starch. II. Properties of the intestinal carbohydrates. *J. Biol. Chem.* **1959**, *233*, 709−726.

(12) De Rijke, E.; Zappey, H.; Ariese, F.; Gooijer, C.; Brinkman, U. A. T. Flavonoids in Leguminosae: analysis of extracts of *T. pratense* L., *T. dubium* L., *T. repens* L., and *L. corniculatus* L. leaves using liquid chromatography with UV, mass spectrometric and fluorescence detection. *Anal. Bioanal. Chem.* **2004**, *378*, 995−1006.

(13) 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.

(14) Ho, H. M.; Chen, R. Y.; Leung, L. K.; Chan, F. L.; Huang, Y.; Chen, Z.-Y. Difference in flavone profile between soybean and soy leaf. *Biomed. Pharmacother.* **2002**, *56*, 289−295.

(15) Lin, L.-Z.; He, X.-G.; Lindenmaier, M.; Yang, J.; Cleary, M.; Qiu, S.-X.; 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.

(16) 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.

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

(18) He, X.-G.; Lin L.-Z.; Lian L.-Z. Analysis of flavonoids from red clover by liquid chromatography-electrospray mass spectrometry. *J. Chromatogr. A* **1996**, *755*, 127−132.

(19) Schutt, D. A. The effect of plant oestrogens on animal reproduction. *Endeavor* **1976**, 35, 110−113.

(20) Dixon, R. A. Phytoestrogens. *Annu. Rev. Plant Biol.* **2004**, *55*, 225−261.

(21) Adlercreutz, H. Phytoestrogens, State of the art. *Environ. Toxicol. Pharmacol.* **1999**, *7*, 201−207.

(22) Franke, A. A.; Custer L. J.; Cerna C. M.; Narala, K. K. Quantitation of phytoestrogens in legumes by HPLC. *J. Agric. Food Chem.* **1994**, *42*, 1905−1913.

Received for review November 30, 2004. Revised manuscript received March 15, 2005. Accepted March 20, 2005.

JF047995F