LC-ESI-MS Study of the Flavonoid Glycoside Malonates of Red Clover (*Trifolium pratense*)

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High-performance liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS) was applied to the analysis of the flavonoids and their glycoside malonates of the flowers and leaves of red clover (*Trifolium pratense*). Through LC-MS comparative studies on the plant extracts and their malonate-free extracts, ~20 flavonoid glycoside malonates were detected in the flower extract. Eight were identified as genistin 6″-*O*-malonate (**39**), formononetin 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**40**), biochanin A 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**41**), trifoside 6″-*O*-malonate (**42**), irilone 4′-*O*-*β*-D-glucoside 6″-*O*-malonate (**43**), pratensein 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**44**), isoquercitrin 6″-*O*-malonate (**45**), and 3-methylquercetin 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**46**). About 15 other flavonoids and clovamides were proved to be present in this extract. The study also found that the flowers contained flavones as the major flavonoids, whereas the leaves had isoflavones as the major flavonoids. This is the first detection of the six malonates (**39** and **42**−**46**) in the extracts of red clover, and among them, **42**, **43**, and **46** are new compounds.

Keywords: *Trifolium pratense; red clover; Leguminosae; flavonoids; flavonoid glycoside malonates; clovamides; LC-ESI-MS analysis*

INTRODUCTION

Trifolium pratense L. (Leguminosae), known as red clover, meadow clover, purple clover, and cow clover, a biennial plant, grows throughout the world. As an important feeding material for sheep and cattle, and a health food for humans, it has estrogenic, antispasmodic, and expectorant properties (Leung et al., 1996). One group of its biologically active components is the flavonoids. To date, >20 flavonoids (Kattaev et al., 1972; Dewick, 1977, 1988; Biggs et al., 1978; Frainshtat et al., 1979, 1980; Jain et al., 1986; Saxena et al., 1987; Harborne, 1994; He et al., 1996), including several malonates (Beck et al., 1971; Edwards et al., 1997), have been reported from this plant. The malonates are of biological interest owing to the ability of the plants to utilize this conjugated form to store the less soluble flavonoid aglycons. Moreover, upon infection of the cell, the aglycons are generated from these precursors by the hydrolysis of the stored form (Sumner et al., 1996; Edwards et al., 1997). When challenged with viral or fungal infection, red clover produces phytolexins, including medicarpin, maackiain, their derivatives, glycosides, and malonates (Edwards et al., 1997; Dewick, 1977, 1988; Harborne, 1994). Two previous LC-MS studies reported the detection of some of the major flavonoids of red clover, but they did not report the malonates of this plant (He et al., 1996; Balogh, 1997). During a comparative LC-MS identification of formononetin 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**40**) in Huangqi (the roots of *Astragalus mongholicus*) with that in red clover (Lin et al., 2000), the existence of ~10 malonates was determined in red clover flowers. This LC-ESI-MS study revealed the existence of >30 flavonoids, including ~20 malonates, in the extract of red clover flowers. The flavonoid glycoside malonates were identified on the basis of the mass spectral and UV data and their conversion to known flavonoid glycosides. Eight malonates were identified as genistin 6″-*O*-malonate (**39**), formononetin 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**40**), biochanin A 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**41**), trifoside 6″-*O*-malonate (**42**), irilone 4′-*O*-*β*-D-glucoside 6″-*O*-malonate (**43**), pratensein 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**44**), isoquercitrin 6″-*O*-malonate (**45**), and 3-methylquercetin 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**46**), respectively. Among them, six of the malonates (**39** and **42**−**46**) were not previously reported from this plant, and three of them (**42**, **43**, and **46**) are new. Their structures are shown in Figure 1. In this paper, we present the detection and identification of these flavonoids of red clover.

MATERIALS AND METHODS

LC-ESI-MS Instrumentation. An HP 1090 Series II LC system (Hewlett-Packard, Palo Alto, CA) with a photodiode array detector set at 260 nm was coupled to an HP 5989B quadrupole mass spectrometer. UV spectra were obtained by scanning from 200 to 500 nm. A Waters SymmetryShield column (5 μm, 2.1 × 150 mm) (Waters Co., Milford, MA) with a sentry guard column (SymmetryShield, 5 μm, 3.9 × 20 mm) was used. The mobile phase consisted of (A) water containing 0.25% acetic acid (v/v) and (B) acetonitrile containing 0.25% acetic acid (v/v), using linear gradients of 14−22% B (v/v) in

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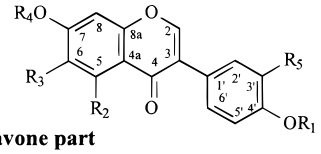
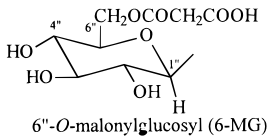
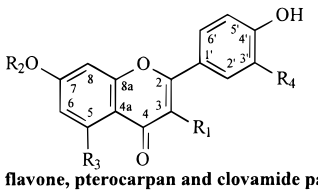




6"-*O*-malonylglucosyl (6-MG)

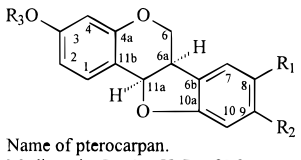
Isoflavone part

Name of isoflavone	R_1	R_2	R_3	R_4	R_5	MW
Daidzein (**1**)	H	H	H	H	H	254
Daidzin (daidzein-7-*O*-β-D-glucoside, **2**)	H	H	H	glc	H	416
Genistein (**3**)	H	OH	H	H	H	270
Genistin (genistein-7-*O*-β-D-glucoside, **4**)	H	OH	H	glc	H	432
Genistin-6"-*O*-malonate (**39**)	H	OH	H	6-MG	H	518
Formononetin (**5**)	Me	H	H	H	H	268
Ononin (formononetin-7-*O*-β-D-glucoside, **6**)	Me	H	H	glc	H	430
Formononetin-7-*O*-β-D-glucoside-6"-*O*-malonate (**40**)	Me	H	H	6-MG	H	516
Biochanin A (**7**)	Me	OH	H	H	H	284
Sissotrin (biochanin A-7-*O*-β-D-glucoside, **8**)	Me	OH	H	glc	H	446
Biochanin A-7-*O*-β-D-glucoside-6"-*O*-malonate (**41**)	Me	OH	H	6-MG	H	532
Calycosin (**9**)	Me	H	H	H	OH	284
Calycosin-7-*O*-β-D-galactoside (**10**)	Me	H	H	gal	OH	446
Calycosin-7-*O*-β-D-glucoside (**20**)	Me	H	H	glc	OH	446
Calycosin-7-*O*-β-D-glucoside-6"-O-malonate (**21**)	Me	H	H	6-MG	OH	532
Prunetin (**11**)	H	OH	H	Me	H	284
Trifoside (prunetin-4'-*O*-β-D-glucoside, **12**)	glc	OH	H	Me	H	446
Trifoside-6"-*O*-malonate (**42**)	6-MG	OH	H	Me	H	532
Glycitein (**24**)	H	H	OMe	H	H	284
Glycitin (glycitein-7-*O*-β-D-glucoside, **25**)	H	H	OMe	glc	H	446
Irilone (**13**)	H	OH	OCH_2-		H	298
Irilone-4'-*O*-β-D-glucoside (**14**)	glc	OH	OCH_2-		H	460
Irilone-4'-*O*-β-D-glucoside-6"-*O*-malonate (**43**)	6-MG	OH	OCH_2-		H	546
Afrormosin (**22**)	Me	H	OMe	H	H	298
Afrormosin-7-*O*-β-D-glucoside (**23**)	Me	H	OMe	glc	H	460
Pratensein (**15**)	Me	OH	H	H	OH	300
Pratensein-7-*O*-β-D-glucoside (**16**)	Me	OH	H	glc	OH	462
Pratensein-7-*O*-β-D-glucoside-6"-*O*-malonate (**44**)	Me	OH	H	6-MG	OH	548
Pseudobaptigenin (**17**)	CH_2-	H	H	H	O-	282
Rothindin (pseudobaptigenin-7-*O*-β-D-glucoside, **18**)	CH_2-	H	H	glc	O-	444
Pectolinarigenin (**19**)	Me	OH	OMe	H	H	314

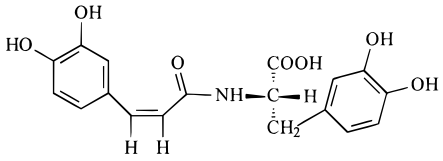


The flavone, pterocarpan and clovamide part

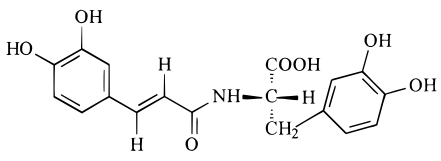
Name of flavone	R_1	R_2	R_3	R_4	MW
Quercetin (**26**)	OH	H	OH	OH	302
Isoquercitrin (quercetin-3-*O*-β-D-glucoside, **27**)	O-glc	H	OH	OH	464
Isoquercitrin-6"-O-malonate (**45**)	6-MG	H	OH	OH	550
Hyperoside (quercetin-3-*O*-β-D-galactoside, **28**)	O-gal	H	OH	OH	464
3-Methylquercetin (**29**)	OMe	H	OH	OH	316
3-Methylquercetin-7-*O*-β-D-glucoside (**30**)	OMe	glc	OH	OH	478
3-Methoxyquercetin-7-*O*-β-D-glucoside-6"-*O*-malonate (**46**)	OMe	6-MG	OH	OH	564
Isorhamnetin (**31**)	OH	H	OH	OMe	316
Luteolin (**32**)	H	H	OH	OH	286
Luteolin-7-*O*-β-D-glucoside (**33**)	H	glc	OH	OH	448
Kaempferol (**34**)	OH	H	OH	H	286
Apigenin (**35**)	H	H	OH	H	270
Apigenin-7-*O*-β-D-glucoside (**36**)	H	glc	OH	H	432



Name of pterocarpan.
Medicarpin: R_1=R_3=H, R_2=OMe, MW=270
Medicarpin-3-*O*-β-D-glucoside: MW=432
6"-*O*-Malonate of medicarpin-3-*O*-β-D-glucoside: MW=518
Maackiain: R_3=H, R_1-R_2=OCH_2O, MW=284
Trifolirhizin: R1-R2=OCH_2O, R3=glucosyl, MW=446
6"-*O*-Malonates of Trifolirhizin: MW=532



Cis-Clovamide (**37**), MW=359



Trans-Clovamide (**38**), MW=359

Figure 1. Structures of the flavones, isoflavones, pterocarpans, and clovamides.

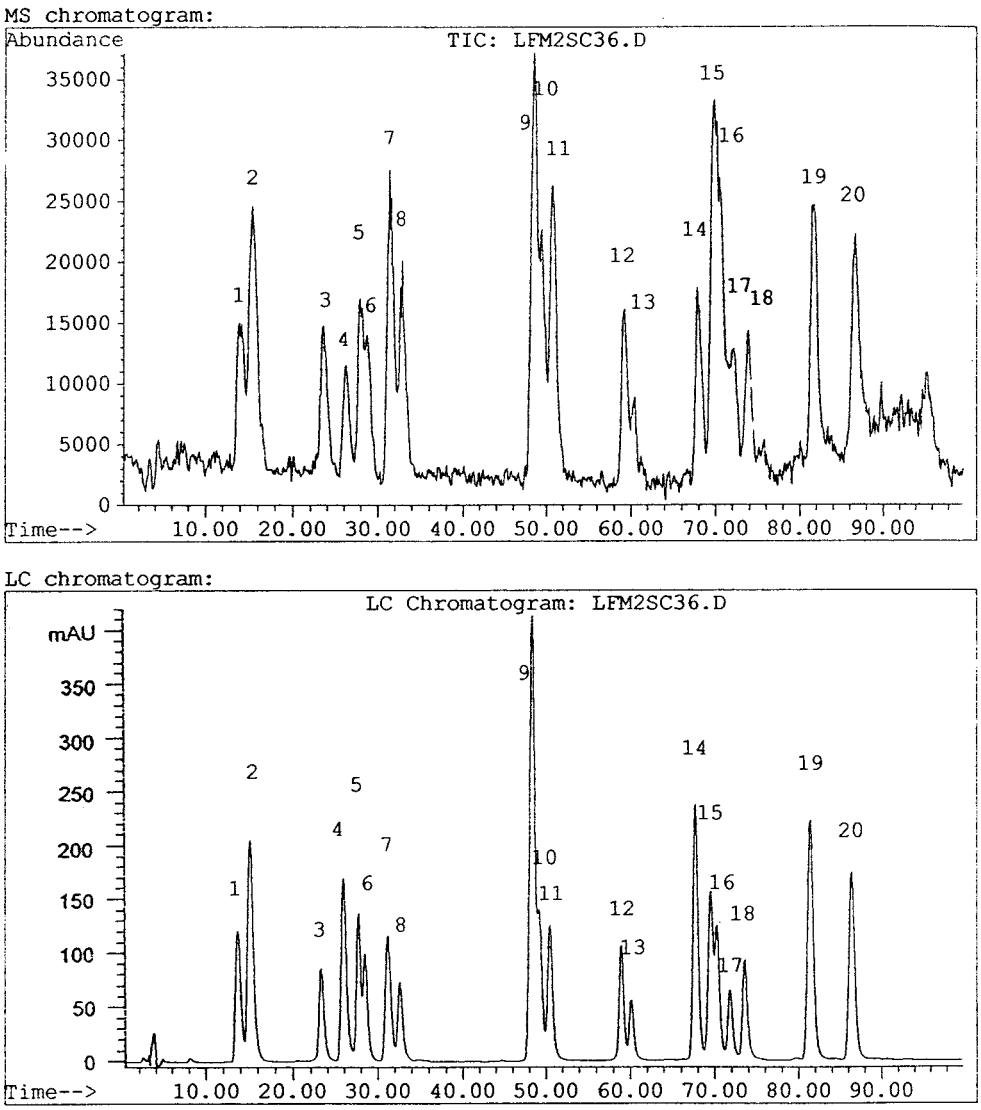


Figure 2. Simultaneous LC-UV and LC-ESI-MS chromatograms of the flavonoid standard compounds. Chromatographic conditions are described under Materials and Methods. The t_R value, MS, and UV λ_{max} of each compound are listed in Table 1.

Table 1. Retention Time (t_R) Value, $[M + H]^+$, Fragment Ions, and UV λ_{max} of the Standard Flavonoids

peak	compound	t_R (min)	$[M + H]^+$ (m/z)	fragment ion (m/z)	λ_{max}[b] (nm)
1	daidzin (**2**)	13.6	417	255	248, 302sh
2A	glycetin (**25**)	15.0[a]	447	285	257, 320
2B	calycosin 7-*O*-*β*-D-glucoside (**20**)	15.0[a]	447	285	260, 290sh
3	luteolin 7-*O*-*β*-D-glucoside (**33**)	23.2	449	287	253, 266sh, 347
4	genistin (**4**)	25.8	433	271	258, 330sh
5	hyperoside (**28**)	27.6[a]	465	303	255, 355
6	isoquercitrin (**27**)	28.4[a]	465	303	255, 355
7	ononin (**6**)	31.1	431	269	253, 303sh
8	apigenin 7-*O*-*β*-D-glucoside (**36**)	32.5	433	271	267, 337
9A	glycetein (**24**)	48.1	285		257, 320
9B	sissotrin (**8**)	48.1	447	285	262, 325sh
10	daidzein (**1**)	49.1	255		248, 302
11	calycosin (**9**)	50.5	285		250, 290
12	luteolin (**32**)	58.9	287		253, 265sh, 347
13	quercetin (**26**)	60.1	303		255, 370
14	genistein (**3**)	67.7	271		260, 330sh
15	formononetin (**5**)	69.5	269		250, 301sh
16	apigenin (**35**)	70.2	271		268, 337
17	isorhamnetin (**31**)	71.6	317		253, 370
18	kaempferol (**34**)	73.5	287		267, 366
19	prunetin (**11**)	81.2	285		262, 325sh
20	biochanin a (**7**)	86.1	285		262, 325sh

[a] Separated by a Waters Symmetry column with the same mobile phase at 10−25% B in 60 min: 17.5 min for glycitin, 22.5 min for calycosin 7-*O*-*β*-D-glucoside; 22.4 min for hyperoside, and 23.9 min for isoquercitrin. [b] For accuracy, the UV values presented are from the LC chromatogram of a well-separated mixture of the standard.

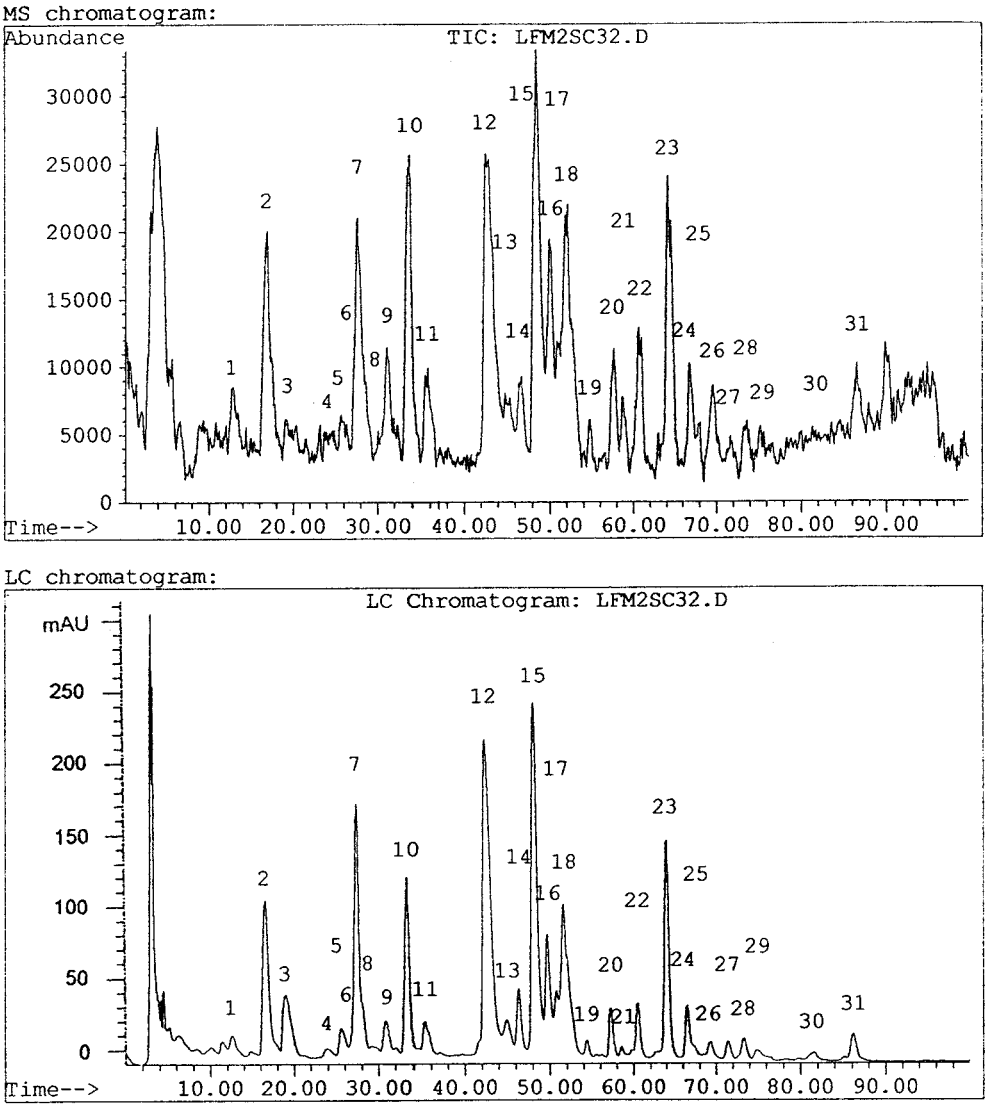


Figure 3. Simultaneous LC-UV and LC-ESI-MS chromatograms of an extract of the flowers of *T. pratense*. Chromatographic conditions are described under Materials and Methods. Peak assignments are listed in Table 2.

36 min, followed by 52% B at 100 min. A Waters Symmetry column with a linear gradient of 10−25% B in 60 min was also used for the separation of glycitin (**25**) (t_R = 17.5 min) and calycosin 7-*O*-*β*-D-glucoside (**20**) (t_R = 22.5 min) and of hyperoside (**28**) (t_R = 22.4 min) and isoquercitrin (**27**) (t_R = 23.9 min) to further confirm their existence in the extracts. The flow rate was 0.2 mL/min, and the temperature of the column oven was 45 °C. The ESI-MS spectra were acquired in the positive ion mode, using an electospray interface model HP 59987A. The temperature of the drying gas (N_2) was 350 °C, at a gas flow rate of 40 mL/min and a nebulizing pressure (N_2) of 5.5×10^5 Pa (80 psi). The LC system was directly connected to the mass spectrometer without stream splitting.

Materials. The botanical materials used in the LC-MS study were the dried fresh flowers and leaves of *T. pratense*, identified and obtained from Meridian Trading Co. (Boulder, CO). Calycosin (**9**) and calycosin 7-*O*-*β*-D-glucoside (**20**) were isolated from *Astragalus mongholicus* in this laboratory (Lin et al., 2000). Daidzein (**1**), genistein (**3**), and biochanin A (**7**) were purchased from Sigma Chemical Co. (St. Louis, MO). All other standards were purchased from Indofine Chemical Co., Inc. (Somerville, NJ). The HPLC solvents and acetic acid were purchased from VWR Scientific (Seattle, WA).

Solution of Standard Compounds. A reference methanolic solution (10 mL) contained **1** (0.25 mg), **2** (0.42 mg), **3** (0.27 mg), **4** (0.43 mg), **5** (0.27 mg), **6** (0.43 mg), **7** (0.28 mg), **8** (0.45 mg), **9** (0.28 mg), **11** (0.28 mg), **20** (0.45 mg), **24** (0.28 mg), **25** (0.45 mg), **26** (0.30 mg), **27** (0.46 mg), **28** (0.46 mg), **31** (0.32 mg), **32** (0.29 mg), **33** (0.45 mg), **34** (0.29 mg), **35** (0.33 mg), and **36** (0.51 mg).

Sample Preparation for LC-MS Study. *Plant Extract Samples.* Dried ground material (0.5 g of the flowers or 0.25 g of the leaves) was extracted with methanol/water (10 mL, 9:1, v/v) using sonication for 60 min at room temperature. The extract was filtered through a 0.45 μm nylon acrodisk 13 filter (Gelman, Ann Arbor, MI). A 10 μL sample of the extract was injected onto the analytical column for analysis. The extraction method appeared to be suitable and effective for the complete extraction of all of the flavonoids, and the sample preparation and analytical conditions were adjusted to prevent degradation of the compounds of interest (Lin et al., 2000).

Malonate-Free Samples. The filtered extract solution (2 mL) was heated in a sealed vial at 80−85 °C for 16 h. After standing at room temperature for 1 h, the solution was filtered in the same way as above for LC injection. This procedure converts almost all of the flavonoid glycoside malonates into their glycosides without the production of acetylglucosyl and other derivatives (Lin et al., 2000).

Hydrolyzed Samples. The filtered extract solution (2.0 mL) was mixed with concentrated HCl (37%, 0.4 mL) and heated in a sealed vial at 80−85 °C for 1.5 h. The solution was refiltered prior to HPLC injection.

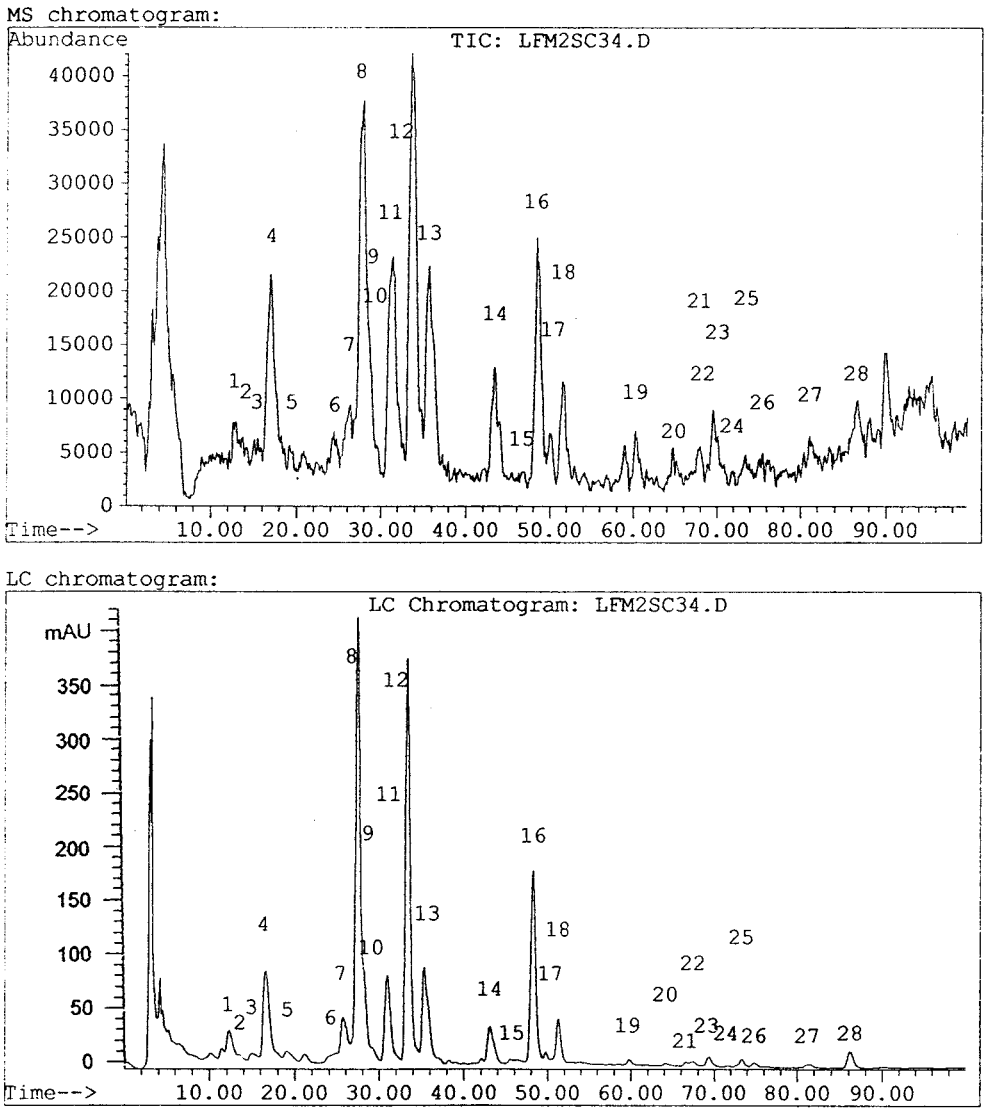


Figure 4. Simultaneous LC-UV and LC-ESI-MS chromatograms of the malonate-free sample of the flowers of *T. pratense*. Chromatographic conditions are described under Materials and Methods. Peak assignments are listed in Table 3.

RESULTS AND DISCUSSION

LC-ESI-MS Analysis of Flavonoid Standards. The solution of flavonoid standards was chromatographed to determine their retention times (t_R), UV, and MS data for comparison with the chromatograms of the plant extracts and other samples. Corresponding LC-UV and total ion chromatograms are shown in Figure 2, and their retention times (t_R) on two different columns, mass, and UV λ_{max} values are listed in Table 1.

To verify the correct identification of the flavonoids in red clover, 22 standards, including several groups of flavonoid isomers, commonly occurring in leguminous plants (Harborne, 1994) were used in this study. They were distinguished by retention time, or $[M + H]^+$ and/or UV λ_{max} values, as listed in Table 1. For example, the four isoflavones biochanin A (5,7-dihydroxy-4′-methoxyisoflavone, **7**), calycosin (7,3′-dihydroxy-4′-methoxyisoflavone, **9**), prunetin (5,4′-dihydroxy-7-methoxyisoflavone, **11**), and glycitein (7,4′-dihydroxy-6-methoxyisoflavone, **24**) are constitutional isomers and were shown to be quite different by their retention times. Among their glycosides, sissotrin (biochanin A 7-*O*-*β*-D-glucoside, **8**) showed good separation from calycosin 7-*O*-*β*-D-glucoside (**20**) and glycetin (glycetein 7-*O*-*β*-D-glucoside, **25**). The latter glucosides were separated using a Waters Symmetry column. Other pairs of isomers, luteolin (5,7,3′,4′-tetrahydroxyflavone, **32**) and kaempferol (3,5,7,4′-tetrahydroxyflavone, **34**), genistein (5,7,4′-trihydroxyisoflavone, **3**) and apigenin (5,7,4′-trihydroxyflavone, **35**), and their glycosides, genistin (genistein 7-*O*-*β*-D-glucoside, **4**) and apigenin 7-*O*-*β*-D-glucoside (**36**), were distinguished from each other by their retention times. Because the UV spectra of the flavonoids are quite different from those of the isoflavonoids (Mabry et al., 1970), flavones **35** and **36** were easily distinguished from the isoflavones **3** and **4** by their UV data. Furthermore, isoquercitrin (quercetin 3-*O*-*β*-D-glucoside, **27**) and hyperoside (quercetin 3-*O*-*β*-D-galactoside, **28**), a pair of quercetin 3-*O*-*β*-D-glycosides consisting of glucose or galactose, differing only by the 4″-hydroxyl orientation of the sugar, were separated under these LC conditions. The separation was optimized using the Waters Symmetry column to provide compound **28** with $t_R = 22.4$ min and **27** with $t_R = 23.9$ min. Thus, the LC-MS conditions used in this study were able to distinguish these standards, and

Table 2. Peak Assignment for the Analysis of the Aqueous Methanol Extract of the Flowers of *T. pratense*

peak	t_R (min)	$[M + H]^+$ (m/z)	fragment ion (m/z)	λ_{max} (nm)	identification
1	12.2	359 (M^+)		294, 328	*trans*-clovamide (**38**)
2	16.5	359 (M^+)		288, 320	*cis*-clovamide (**37**)
3	19.0	*a*	*a*	294, 328	caffeic acid derivative
4	23.9	*a*	*a*	290, 320	caffeic acid derivative
5	24.3	463	291	262, 320sh	pratensein 7-*O*-*β*-D-glucoside (**16**)
6	25.8	433	271	258, 330sh	genistin (**4**)
7	27.6	465	303	255, 355	hyperoside (**28**)
8	28.4	465	303	255, 355	isoquercitrin (**27**)
9	31.1	431	269	253, 303sh	ononin (**6**)
10	33.2	449	287	265, 347	unknown tetrahydroxyflavone glucoside
11A	35.3	479	317	255, 355	3-methylquercetin 7-*O*-*β*-D-glucoside (**30**)
11B	~36.0	449	287	*a*	unknown tetrahydroxyflavone glucoside
12A	42.9	551	303	255, 355	isoquercitrin 6″-*O*-malonate (**45**)
12B	42.9	461	299	*a*	irilone 4′-*O*-*β*-D-glucoside (**14**)
13A	44.9	535	287	265, 347	unknown malonate (related to peak 10)
13B	44.9	551	303	*a*	unknown malonate (isomer of **45**)
14A	46.3	549	301	262, 320sh	pratensein 7-*O*-*β*-D-glucoside 6″-malonate (**44**)
14B	46.3	519	271	*a*	genistin 6″-*O*-malonate (**39**)
14C	46.3	461	299	*a*	afrormosin 7-*O*-*β*-D-glucoside (**23**)
15A	48.3	535	287	265, 347	unknown malonate (related to peak 10)
15B	48.3	447	285	*a*	sissotrin (**8**)
16	49.6	565	317	255, 355	3-methylquercetin 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**46**)
17	50.7	535	287	265, 347	unknown malonate (related to peak 11B)
18A	51.5	517	269	253, 303sh	formononetin 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**40**)
18B	51.5	447	285	262, 325sh	trifoside (**12**)
18C	~52.0	535	287	*a*	unknown malonate
18	~52.2	551	303	*a*	unknown malonate
19	54.4	565	317	255, 355	unknown malonate (isomer of **46**)
20A	57.2	517	269	*a*	unknown malonate (isomer of **40**)
20B	57.2	535	287	265, 347	unknown malonate (related to peak 11b)
21	58.5	565	317	*a*	unknown malonate
22	60.4	547	299	270, 305sh	irilone 4′-*O*-*β*-D-glucoside 6″-*O*-malonate (**43**)
23	63.9	533	285	262, 325sh	biochanin A 7-*O*-*β*-D-glucoside 6″-*O*-malonate (**41**)
24	66.5	533		262, 325sh	unknown malonate (isomer of **41**)
25	67.7	271		*a*	genistein (**3**)
26	69.5	269		250, 301sh	formononetin (**5**)
27	71.4	533	285	262, 325sh	trifoside 6″-*O*-malonate (**42**)
28A	73.2	533	285	262, 325sh	unknown malonate (isomer of **42**)
28B	~73.5	287		*a*	unknown tetrahydroxyflavone
29	74.7	299		*a*	irilone (**13**) or afrormosin (**22**)
30	81.2	285		*a*	prunetin (**11**)
31	86.1	285		262, 325sh	biochanin a (**7**)

[a] Unable to measure.

consequently, the conditions could be utilized to separate and identify the flavonoids in red clover.

LC-ESI-MS Analyses of the Extract and Its Malonate-Free Sample of the Flowers of *T. pratense*. The LC and total ion chromatograms of the flower extract and its malonate-free sample are shown in Figures 3 and 4, and the retention time (t_R), mass, and UV λ_{max} values and the identifications for the individual peaks are listed in Tables 2 and 3, respectively. The peak identification (Table 3) of the malonate-free sample was made on the basis of the comparison of the values with those of the standards and published information concerning the flavonoids of red clover. Thus, daidzin (daidzein 7-*O*-*β*-D-glucoside, **2**), genistin (**4**), hyperoside (**28**), isoquercitrin (**27**), ononin (formononetin 7-*O*-*β*-D-glucoside, **6**), sissotrin (**8**), quercetin (**26**), genistein (**3**), formononetin (**5**), prunetin (**11**), and biochanin A (**7**) were identified by comparison of the retention times and mass data with those of the standard samples. Some of these identifications were also confirmed by the UV data. These flavonoids were previously reported in red clover (Frainshtat et al., 1980; He et al., 1996). Peak 17 showed its $[M + H]^+$ at m/z 491 and a fragment ion at m/z 329 (for aglycon), with UV absorptions at 265 and 325sh nm, suggesting it to be the glycoside of an isoflavone containing one hydroxy and three methoxy groups. Such a compound was structurally unknown and not previously reported for red clover. The analysis of the mass and UV data led to the identification of peaks 6, 13A, 14, and 18 as pratensein 7-*O*-*β*-D-glucoside (**16**) (Frainshtat et al., 1980), 3-methylquercetin 7-*O*-*β*-D-glucoside (**30**) (Jain et al., 1986), irilone 4′-*O*-*β*-D-glucoside (**14**) (Frainshtat et al., 1979), and trifoside (prunetin 4′-*O*-*β*-D-glucoside, prunitrin, **12**) (Kattaev et al., 1972), respectively. These glycosides or their corresponding aglycons were isolated from red clover. According to the corrected nomenclature, 3-methylquercetin 7-*O*-*β*-D-glucoside (**30**) is used in this paper to replace the previous name of 3-methoxyquercetin 7-*O*-*β*-D-glucoside (Jain et al., 1986). On the basis of the mass data (Frainshtat et al., 1979, 1980; Jain et al., 1986), peaks 10, 20, 22, 24, and 26 were identified as rothindin (pseudobaptigenin 7-*O*-*β*-D-glucoside, **18**), pratensein (5,7,3′-trihydroxy-4′-methoxyisoflavone, **15**), pseudobaptigenin (7-hydroxy-3′,4′-methylenedioxyisoflavone, **17**), 3-methylquercetin (**29**), and irilone (5,4′-dihydroxy-6,7-methylenedioxyisoflavone, **13**) or afrormosin (7-hydroxy-6,4′-dimethoxyisoflavone, **22**), respectively. Because such identifications were made on the basis of mass spectral data only, which cannot distinguish a compound from its isomer, both of them, for example, **13** and **22** were listed together for the identification of peak 26 in Table

Table 3. Peak Assignment for the Analysis of the Malonate-Free Aqueous Methanol Extract of the Flowers of *T. pratense*

peak	t_R (min)	$[M + H]^+$ (m/z)	fragment ion (m/z)	λ_{max} (nm)	identification
1	12.2	359 (M^+)		294, 328	*trans*-clovamide (**38**)
2	13.6	417	255	*a*	daidzin (**2**)
3	15.0	447	285	*a*	calycosin glucoside (**20**) or galactoside (**10**)
4	16.5	359 (M^+)		288, 320	*cis*-clovamide (**37**)
5	19.0	*a*		294, 328	caffeic acid derivative
6	24.3	463	301	262, 320sh	pratensein 7-*O*-*β*-D-glucoside (**16**)
7	25.8	433	271	258, 330sh	genistin (**4**)
8	27.6	465	303	255, 355	hyperoside (**28**)
9	28.4	465	303	255, 355	isoquercitrin (**27**)
10	29.5	445	283	*a*	rothindin (**18**)
11	31.1	431	269	253, 303sh	ononin (**6**)
12	33.2	449	287	265, 347	unknown tetrahydroxyflavone glucoside
13A	35.3	479	317	255, 355	3-methylquercetin 7-*O*-*β*-D-glucoside (**30**)
13B	~36.0	449	287	*a*	unknown tetrahydroxyflavone glucoside
14	42.9	461	299	270, 305sh	irilone 4′-*O*-*β*-D-glucoside (**14**)
15	46.6	461	299	*a*	afrormosin 7-*O*-*β*-D-glucoside (**23**)
16	48.3	447	285	262, 325sh	sissotrin (**8**)
17	49.7	491	329	265, 325sh	unknown trimethoxyhydroxyisoflavone glucoside
18	51.5	447	285	262, 325sh	trifoside (**12**)
19	60.1	303		*a*	quercetin (**26**)
20	64.0	301		*a*	pratensein (**15**)
21	67.7	271		*a*	genistein (**3**)
22	68.3	283		*a*	pseudobaptigenin (**17**)
23	69.5	269		250, 301sh	formononetin (**5**)
24	70.7	317		*a*	3-methylquercetin (**29**)
25	73.5	287		*a*	unknown tetrahydroxyflavone
26	74.7	299		*a*	irilone (**13**) or afrormosin (**22**)
27	81.2	285		*a*	prunetin (**11**)
28	86.1	285		262, 325sh	biochanin a (**7**)

[a] Unable to measure.

Table 4. Peak Assignment for the Analysis of the Aqueous Methanol Extract of the Leaves of *T. pratense*

peak	t_R (min)	$[M + H]^+$ (m/z)	fragment ion (m/z)	λ_{max} (nm)	identification
1	16.5	359 (M^+)		288, 320	*cis*-clovamide (**37**)
2	19.0	*a*		294sh, 328	caffeic acid derivative
3	24.3	463	301	262, 320sh	pratensein 7-*O*-*β*-D-glucoside (**16**)
4	25.8	433	271	258, 330sh	genistin (**4**)
5	27.6	465	303	255, 355	hyperoside (**28**)
6	28.4	465	303	255, 355	isoquercitrin (**27**)
7	31.1	431	269	253, 303sh	ononin (**6**)
8	33.2	449	287	265, 347	unknown tetrahydroxyflavoneglucoside
9	36.2	449	287	265, 347	unknown tetrahydroxyflavoneglucoside
10A	42.9	551	303	257, 355	isoquercitrin 6″-*O*-malonate (**45**)
10B	42.9	461	299	*a*	irilone 4′-*O*-*β*-D-glucoside (**14**)
11A	46.3	549	301	262, 320sh	pratensein 7-*O*-*β*-D-glucoside 6″-malonate (**44**)
11B	46.3	519	271	*a*	genistin 6″-*O*-malonate (**39**)
12	48.3	447	285	262, 325sh	sissotrin (**8**)
13A	51.5	517	269	253, 303sh	formononetin 7-*O*-*β*-D-glucoside 6″-malonate (**40**)
13B	51.5	447	285	*a*	trifoside (**12**)
14	57.2	517	269	253, 303sh	unknown malonate (isomer of **40**)
15	60.4	547	299	270, 305sh	irilone 4′-*O*-*β*-D-glucoside 6″-malonate (**43**)
16	63.9	533	285	262, 325sh	biochanin A 7-*O*-*β*-D-glucoside 6″-malonate (**41**)
17	66.5	533	285	262, 325sh	unknown malonate (isomer of **41**)
18	69.5	269		250	formononetin (**5**)
19	71.4	533	285	262, 325sh	trifoside 6″-*O*-malonate (**42**)
20	73.2	533	285	262, 325sh	unknown malonate (isomer of **42**)
21	74.7	299		*a*	irilone (**13**) or afrormosin (**22**)
22	82.2	285		*a*	prunetin (**11**)
23	86.1	285		262, 325sh	biochanin A (**7**)

[a] Unable to measure.

3. Peak 15 (a minor peak) was identified as aformosin 7-*O*-*β*-D-glucoside (**23**), an isomer of irilone 4′-glucoside (**14**). Compound **23** and its aglycon (**22**) were reported in several leguminous plants (Harborne, 1994). There are two other flavonoid peaks (12, t_R = 33.2 min; 13B, t_R = 36 min) that showed the same mass (449 and 287 amu) and UV (265 and 347 nm) data for the glycosides of tetrahydroxyflavone, but which were different from luteolin 7-*O*-*β*-D-glucoside (**33**) (t_R = 23.2 min). Peak 25 (t_R = 73.5 min) showed its $[M + H]^+$ at m/z 287 for their aglycon. Because the isolation of pratoletin, a structurally unknown tetrahydroxyflavone, was reported previously from this plant (Chadha, 1965; Harborne, 1994), the two peaks, or at least the major one (peak 12), might be the glycosides of pratoletin. However, such structures cannot be further refined by LC-MS detection alone, and thus the identification of those peaks is indicated as unknown tetrahydroxyflavone and its glycosides.

Table 5. Peak Assignment for the Analysis of the Malonate-Free Aqueous Methanol Extract of the Leaves of *T. pratense*

peak	t_R (min)	$[M + H]^+$ (m/z)	fragment ion (m/z)	λ_{max} (nm)	identification
1	12.2	359 (M^+)		294, 328	*trans*-clovamide (**38**)
2	13.6	417	255	*a*	daidzin (**2**)
3	15.0	447	285	*a*	calycosin glucoside (**20**) or galactoside (**10**)
4	16.5	359 (M^+)		288, 320	*cis*-clovamide (**37**)
5	19.0	*a*	*a*	294, 328	caffeic acid derivative
6	24.3	463	301	262, 320sh	pratensein 7-*O*-β-D-glucoside (**16**)
7	25.8	433	271	258, 330sh	genistin (**4**)
8	27.6	465	303	255, 355	hyperoside (**28**)
9	28.4	465	303	255, 355	isoquercitrin (**27**)
10	29.5	445	283	*a*	rothindin (**18**)
11	31.1	431	269	253, 303sh	ononin (**6**)
12	33.2	449	287	265, 347	unknown tetrahydroxyflavone glucoside
13	36.2	449	287	265, 347	unknown tetrahedroxyflavone glucoside
14	42.9	461	299	270, 305sh	irilone 4′-*O*-β-D-glucoside (**14**)
15	46.6	461	299	*a*	afrormosin 7-*O*-β-D-glucoside (**23**)
16	48.3	447	285	262, 325sh	sissotrin (**8**)
17	51.5	447	285	262, 325sh	trifoside (**12**)
18	54.0	473	312	*a*	unknown flavonoid
19	67.7	271		*a*	genistein (**3**)
20	68.3	283		*a*	pseudobaptigenin (**17**)
21	69.5	269		250, 301sh	formononetin (**5**)
22	81.2	285		*a*	prunetin (**11**)
23	86.1	285		262, 325sh	biochanin A (**7**)

[a] Unable to measure.

It is worth mentioning that in this study each of the flavonoid glycosides displayed not only its $[M + H]^+$ but also a fragment ion which is 162 amu less than the molecular ion of the glycoside due to the loss of the glycosyl group. This ion is always important for a direct determination of the molecular weight of the aglycon present in the glycoside.

There were still three remaining peaks showing UV data different from those of the peaks for the flavonoids. Peak 1 with its $[M]^+$ at m/z 359, UV at 294 and 328 nm, was identified as *trans*-clovamide (**38**), whereas peak 4 was identified as *cis*-clovamide (**37**) on the basis of the same mass data and its UV maxima at 288 and 320 nm (Yoshihara et al., 1977). Peak 5 should be a caffeic acid derivative (Yoshihara et al., 1977), showing a UV spectrum similar to that of *trans*-clovamide (**38**), but it did not offer a significant mass ion peak under these LC-MS conditions to permit its complete identification.

Comparison of the LC-MS chromatogram (Figure 3) of the extract with that (Figure 4) of its malonate-free sample indicated that the relative peak area ratios of the peaks in these two chromatograms were quite different. There are a number of peaks in the range of 40−74 min in Figure 3, which disappeared after the 16 h heating process and were converted into their related glycosides. As a result, some peaks in Figure 4 at the ranges of 26−40 and 48−52 min were substantially increased. All of these peaks in Figure 3 showed their $[M + H]^+$ ions at >500 amu and fragment ions for the aglycons, which were 248 amu less than those of the molecular mass ions of the parent compounds. This value (248 amu) is 86 amu larger than that of the glucosyl group (162 amu) of the glycoside, and 86 amu corresponds to a malonoyl group ($OCCH_2COOH$). Furthermore, during the heating process, the malonate lost its malonyl ester and was converted into its related glycoside. Both the malonate and its related glycoside showed the same UV data, which suggested that the malonyl group was not attached to any of the phenolic functions at the aglycon part (Mabry et al., 1970). The above facts led to the conclusion that the malonyl group should be attached only to the glycosyl part of the molecule. This conclusion is consistent with the structures of formononetin 7-*O*-β-D-glucoside 6″-*O*-malonate (**40**) and biochanin A 7-*O*-β-D-glucoside 6″-*O*-malonate (**41**), which were established by spectral analysis of the pure compounds from this plant (Beck et al., 1971). Thus, peaks 12A, 14A, 14B, 16, 18A, 22, 23, and 27 (Figure 3) were identified as isoquercitrin 6″-*O*-malonate (quercetin 3-malonylglucoside, **45**) (Asen, 1984; Gluchoff-Fiasson et al., 1997), pratensein 7-*O*-β-D-glucoside 6″-*O*-malonate [pratensein 7-*O*-β-(6″-malonyl)-glucoside, **44**] (Anhut et al., 1984; Stein et al., 1985), genistin 6″-*O*-malonate (6″-*O*-malonylgenistin, **39**) (Kudou et al., 1991; Park et al., 1995; Stobiecki et al., 1999), 3-methylquercetin 7-*O*-β-D-glucoside 6″-*O*-malonate (**46**), formononetin 7-*O*-β-D-glucoside 6″-*O*-malonate (ononin 6″-*O*-malonate, malonylononin, **40**), irilone 4′-*O*-β-D-glucoside 6″-*O*-malonate (**43**), biochanin A 7-*O*-β-D-glucoside 6″-*O*-malonate (**41**), and trifoside 6″-*O*-malonate (**42**), respectively. Their structures are shown in Figure 1. Except for **40** and **41** (Beck et al., 1971; Edwards et al., 1996), this is the first detection of six other malonates (**39** and **42**−**46**) in red clover, and three of them (**42**, **43**, and **46**) are new malonates. The structures of the new malonates were assigned to have the same 6″-*O*-malonylglucosyl as that of the coexisting malonates **40** and **41** on the basis of the consideration that they probably might be formed through the same biogenetic pathway. This assignment is also supported by the fact that, so far, all of the flavonoid glycoside malonates from plants, including **39**, **44**, and **45**, have a 6″-*O*-malonylglucosyl unit (Dewick, 1988; Harborne, 1994).

It is worth mentioning that there are five minor peaks (13B, 19, 20A, 24, and 28A), each of them (for example, peak 19) showing the same mass and UV data as those of one of the above malonates (peak 6 for malonate **46**, the major malonate of 3-methylquercetin 7-*O*-β-D-glucoside). These peaks were converted to the same related glycoside (**30**) during the heating process. Thus, on the basis of these facts, such a minor malonate could be considered to be an isomer in which the malonyl group

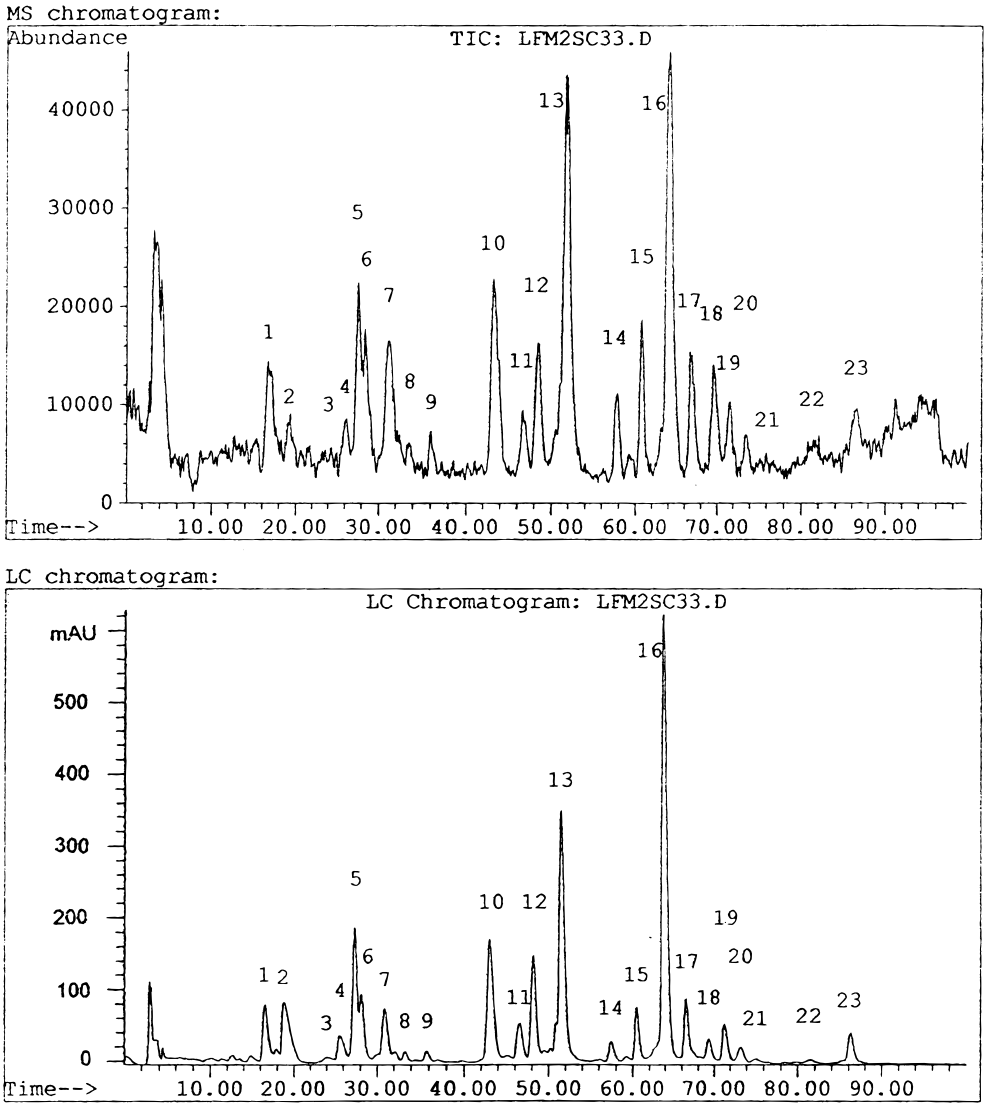


Figure 5. Simultaneous LC-UV and LC-ESI-MS chromatograms of an extract of the leaves of *T. pratense*. Chromatographic conditions are described under Materials and Methods. Peak assignments are listed in Table 4.

was attached at a position other than the 6″-position on the glycosyl moiety. Edwards et al. (1997) reported that biochanin A 7-*O*-*β*-D-glucoside (**8**) formed two forms (BGM2 and one of BGM3 and 4) of malonates in red clover. We also found the coexistence of a pair of malonates for ononin (**6**) and calycosin 7-*O*-*β*-D-glucoside (**20**) in *A. mongholicus* (Lin et al., 2000). To date, there are no reports describing the details of the structures of such minor malonates. Thus, they were indicated as unknown malonates in this paper. In the same way, peaks 13A and 15A, as well as 17 and 20B, seem to be the pairs of the malonates of two unknown tetrahydroxyflavoneglucosides (peaks 10 and 11B). There are three other small peaks (18C, 18D, and 21) that are known to be malonates, but owing to the difficulty of finding their converted glycosides present at the trace level in such a complex solution, they could not be identified. Because this chromatogram (Figure 3) showed the peaks in these regions to be very close and their overlap so severe, some other malonates and flavonoids, especially minor ones, might have been missed in this analysis.

In addition to the mentioned malonates, flavonoids **3**−**8**, **12**−**14**, **16**, **22**, **23**, **27**, **28**, and **30** and two tetrahydroxyflavone glucosides, as well as *cis*- and *trans*-clovamides (**37** and **38**), were also detected in this extract. Because both chromatograms (Figures 3 and 4) showed a broad overlapped peak for hyperoside (**28**) and isoquercitrin (**27**), these samples were reanalyzed using Waters Symmetry column conditions, in which they were separated as two peaks. The peak for **28** was nearly 3 times larger than that for **27**; thus, hyperoside (**28**) is demonstrated to be a probable constituent of red clover flowers (He et al., 1996). In Tables 1−5 and Figures 2−6, for a number of compounds having a difference among their retention times within 1 min, their LC peaks and total mass ion peaks always overlapped to form one broad peak. Thus, to distinguish these compounds, the peak number was suffixed with -A or -B (for example, 13A and 13B in Table 2) for each individual compound. In such cases, the overlapped total mass ion peak provided appropriate mass data for each individual compound, but the overlapped LC peak did not offer the opportunity to measure the individual UV spectrum for each of the compounds involved.

LC-ESI-MS of the Hydrolyzed Extract of the Flowers of *T. pratense*. An LC-MS study was also carried out with the hydrolyzed sample of the flowers to identify the aglycons and to confirm the identification of the glycosides. Analyzed in the same way, the

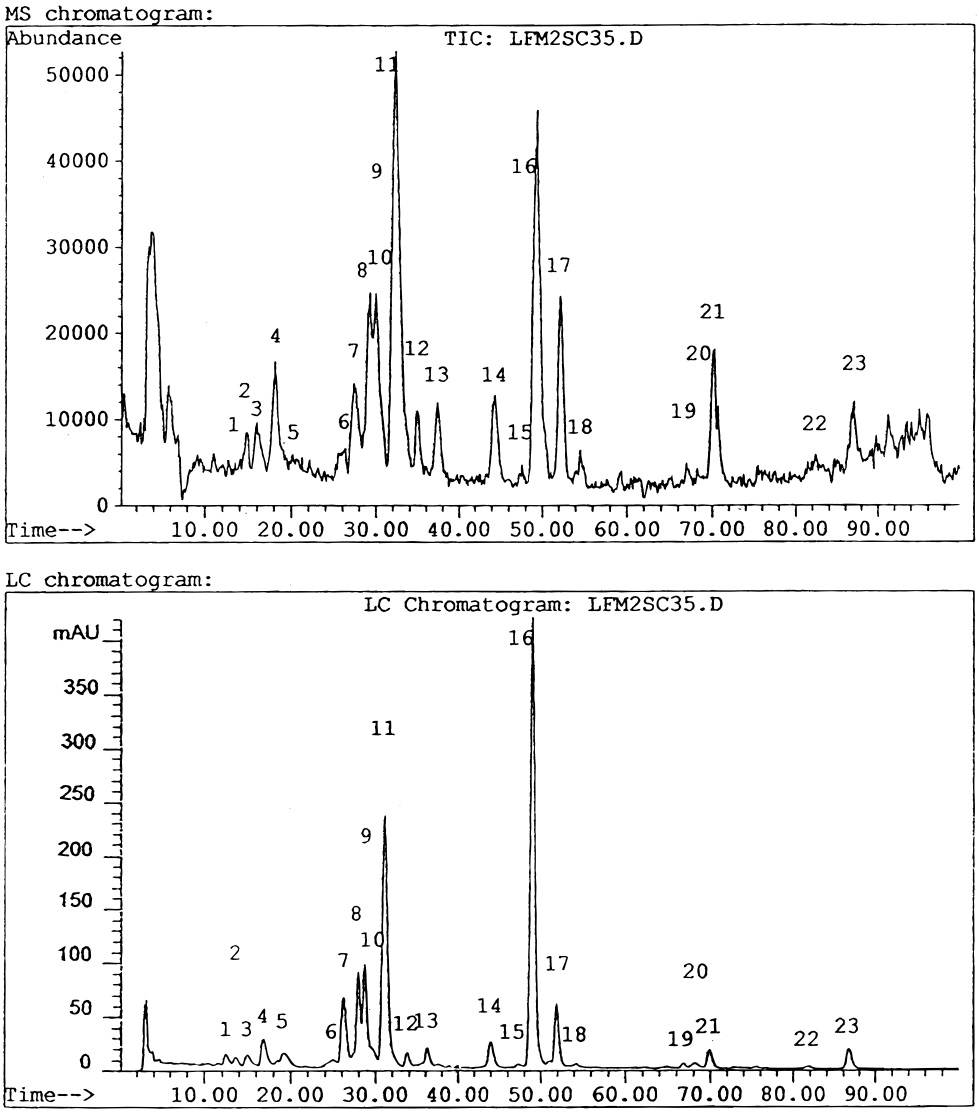


Figure 6. Simultaneous LC-UV and LC-ESI-MS chromatograms of the malonate-free sample of the leaves of *T. pratense*. Chromatographic conditions are described under Materials and Methods. Peak assignments are listed in Table 5.

identified aglycons yielded from the hydrolysis were daidzein (**1**), quercetin (**26**), calycosin (**9**), genistein (**3**), and irilone (**13**) or afrormosin (**22**), pratensein (**15**), formononetin (**5**), 3-methylquercetin (**29**), prunetin (**11**), and biochanin A (**7**). The peak with a retention time at 73.5 min was identified as the aglycon (probably, pratoletin) of the unknown tetrahydroxyflavone glucosides. This aglycon showed the same retention time as kaempferol (**34**) but different from that of luteolin (**32**) (t_R = 58.9 min), suggesting that the unknown tetrahydroxyflavone might be kaempferol (trifolotin, **34**). However, final confirmation still needs to be made by its structural determination through an NMR study because it is difficult to find standards to carry out a complete comparative study with the eight other tetrahydroxyflavones from plants (Harborne, 1994). 3-Methylquercetin (**29**), the isomer of isorhamnetin (**31**), did not show a peak identical with the standard **31**.

On the basis of the fact that the glycosides were converted into their aglycons after hydrolysis, the amount of prunetin (**11**) increased substantially in the hydrolyzed sample, which served to confirm the identification of trifoside (prunutrin, **12**), a commercially unavailable glucoside of **11**. In the same way, the identification of pratensein 7-*O*-β-D-glucoside (**16**), irilone 4′-*O*-β-D-glucoside (**14**), and some other glycosides was also further confirmed by the production of their aglycons in the hydrolyzed sample.

LC-ESI-MS Analyses of the Extract and Its Malonate-Free Sample from the Leaves of *T. pratense*. The LC and total ion chromatograms of the extract and malonate-free sample of the leaves are shown in Figures 5 and 6, and the retention time (t_R), $[M + H]^+$, UV λ_{max} values and the identification for the individual peaks are listed in Tables 4 and 5, respectively. The peak identification of the leaf samples was made on the basis of the comparison of these values with those from red clover flowers (Tables 2 and 3).

The study found that the isoflavonoids sissotrin (**8**) (the peak area for peak 16, 34.0%) and ononin (**6**) (peak 11, 21.8%) formed the two biggest peaks (>50% of the total peak area) on the LC chromatograms (Figure 6 and Table 5) of the malonate-free sample of the leaves. This result is quite different from that of its corresponding flower sample, in which the flavonoids hyperoside (**28**) and quercitrin (**27**) (peaks 8 and 9 overlapped, 28.7%) and the unknown tetrahydroxyflavone glucoside (peak 12, 21.6%) formed the most substantial peaks

(nearly 50% of total peak area) (Figure 4 and Table 3). Thus, the peak areas for sissotrin (**8**, peak 16) and ononin (**6**, peak 11) were only 10.8 and 6.3% in the chromatogram of this flower sample, respectively. The two isoflavones (**6** and **8**) exist in the leaf extract at >50% of the total amount; consequently, some of the minor flavones, for example, 3-methylquercetin (**29**) and its glycoside (**30**), could not be detected because their concentrations were below the limitation of detection (Lin et al., 1998). Furthermore, the chromatograms (Figures 5 and 6) of the leaf samples showed well-separated peaks for hyperoside (**28**, peak area around 6.3% for both peak 5 of Figure 5 and peak 8 of Figure 6) and isoquercitrin (**27**, peak area of 8.9% for peak 9 of Figures 6 and 3.4% for peak 6 of Figure 5). On the basis of the change of these peak areas before and after the conversion from the malonate into the glycoside, it was found that the only significant enhancement (from 3.4 to 8.9% for **27**) was with isoquercitrin (**27**). This suggested that quercetin glycoside malonate in red clover was mainly formed from isoquercitrin (**27**), the glycoside consisting of glucose. The leaf samples also showed a peak for the glycoside of calycosin (**9**), which was identified as probably calycosin 7-*O*-*β*-D-glucoside (**20**), rather than calycosin 7-*O*-*β*-D-galactoside (**10**), on the basis of the same retention time as that of standard **20**. Even though only the isolation of **10** was reported previously (Saxena et al., 1987), it is reasonable to identify this peak as **10** unless both isomers had the same retention time.

Flavonoid glycoside malonates were also reported in several other leguminous plants, including subterranean clover (*Trifolium subterranneum*) (Beck et al., 1971), white clover (*T. repens*) (Cook et al., 1995), alfalfa (*Medicago sativa*) (Coronado et al., 1995), chickpea (*Cicer arietinum*) (Mackenbrock et al., 1992), soybean (*Glycine max*) (Kudou et al., 1991; Barnes et al., 1994), and kudzu (*Pueraria lobata*) (Park et al., 1995). In addition to malonates, soybean was also reported to contain some 6″-*O*-acetylglucosylisoflavoned, which might be formed from the related malonates by loss of carbon dioxide (Horowitz et al., 1989; Kudou et al., 1991; Barnes et al., 1994). However, this LC-MS study with red clover did not find any 6″-*O*-acetylglucosylflavonoids existing in the extracts at a detectable concentration.

This study showed that the method consisting of the LC-MS analysis of both the original extract of the plant and its malonate-free sample was a convenient, suitable, and efficient way to identify the flavonoid glycoside malonates in a plant such as red clover that contains a substantial level of malonates. This study also showed that the LC-ESI-MS technique was a suitable and sensitive method to identify such polar and thermally labile flavonoid glycoside malonates in the plant extract. This technique could offer a correct mass profile, especially the $[M + H]^+$ ion, for the identification of these conjugates and other flavonoids (Barnes et al., 1994; Stobiecki et al., 1999; Lin et al., 2000).

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