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Determination of isoflavones in red clover and related species by high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection

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Abstract

High-performance liquid chromatography–UV–electrospray ionization-mass spectrometric detector (HPLC–UV–ESI-MSD) method for determination of isoflavones in red clover (*Trifolium pratense* L.) and related species has been developed. The separated isoflavones including aglycones, glycosides and glycoside malonates, were individually analyzed and identified by their molecular ions and characteristic fragment ion peaks using LC–MSD under MS and MS–MS mode, and in comparison with the standard isoflavones. A total of 31 isoflavones were detected in red clover. Several isoflavones were also identified for the first time in related species, *T. repense* L. (white clover), *T. hybridum* L. (alsike clover) and *T. campestre* Schreber (hop trefoil). Based on reversed phase HPLC, all 10 isoflavone aglycones, daidzein, formononetin, genistein, pseudobaptigenin, glycitein, calycosin, prunetin, biochanin A, irilone and pratensein in acidic hydrolyzed extracts were successfully separated within 40 min and quantified individually by UV and MS detectors. For the 10 target compounds, the investigated concentrations ranged from ~24 to ~12500 ng/ml for UV detection and ~6 to ~3125 ng/ml for MS detection, and good linearities ($r^2 > 0.999$ for UV and $r^2 > 0.99$ for MS) for standard curves were achieved for each isoflavone. The accuracy and repeatability ($n = 10$) were within 15% for these 10 compounds. This is the first method reported that enables the simultaneous quantitation of all 10 isoflavone aglycones in red clover and related species.

Keywords: Red clover; *Trifolium pratense* L.; Isoflavones

1. Introduction

Natural isoflavones widely distributed in Leguminosae family are plant chemicals with estrogenic activity, and represent the main class of phytoestrogens of current interest in clinical nutrition [1–4]. Isoflavone phytoestrogens based on the structural similarity to the estrogens 17β-estrodiol (Fig. 1) have been found to exert potential health benefits in age-related and hormone dependent diseases, including cancer, menopausal symptoms, cardiovascular disease and osteoporosis [1,2]. Red clover (*Trifolium pratense* L.), a legume and an important forage plant grown around the world contains many of the same

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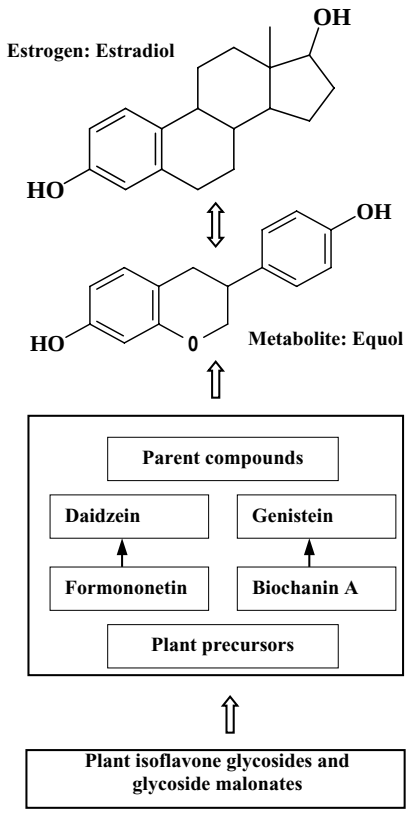


Fig. 1. Metabolic pathway of plant isoflavones in humans and animals, and comparison of the structure of the main isoflavone metabolite equol with that of estradiol showing a marked similarity.

isoflavones long recognized and extensively studied in soy, including aglycones and their glycoside and glycoside malonate derivatives [5–10]. Red clover was originally used as a medicinal herb by native indigenous people of North America for whooping cough, gout and cancer [11], and others traditionally have used it in the treatment of asthma, bronchitis, coughs, athlete's foot [12] and for eczema, psoriasis [13].

Extracts of red clover are commercially available as isoflavone enriched dietary supplements on the US and European markets for women suffering under menopausal complaints [13,14]. As a consequence, there is an increasing demand for new supplements and alternative isoflavone sources beyond soy, such as red clover. In red clover, the majority of the aglycones are formononetin and biochanin A, with smaller concentrations of daidzein and genistein, the major phytoestrogens found in soy products. Several methods to characterize the content of isoflavones in red clover using liquid chromatography combined with ultraviolet and/or electrospray ionization–mass spectrometric detector have been reported [9,10,13,15–17]. Qualitative studies using red clover leaf and flower only led to the identification of numerous isoflavones [9,10]. Other quantitative studies using HPLC methods of analysis focused on determination of the original or acidic hydrolyzed fractions of red clover extracts. However, only part of target components, formononetin, biochanin A, daidzein, genistein and their glycosides and glycoside malonates were quantified, and the researchers were not able to determine accurate levels of the total isoflavones in red clover [13,15–17]. The purpose of this research was to study the isoflavonoid profile and the total isoflavones in different parts (leaf, stem, root and flower) of red clover and to establish a more robust and inclusive analytical method providing both a qualitative and quantitative method for the isoflavones in red clover and related species. Under optimized conditions, we were able to simultaneously identify 31 isoflavones including 9 aglycones, 8 glycosides and 14 glycoside malonates from red clover. As pratensein only occurs as a glycoside except under hydrolyzed conditions where it forms an aglycone, this method allowed all 10 isoflavone aglycones in acidic hydrolyzed red clover extracts to be successfully quantified individually using RP-HPLC with UV and MS detector. To search for additional new plant isoflavone sources in *Trifolium* genus, four tissues of three other commonly available species, *T. repense* L. (white clover), *T. hybridum* L. (alsike clover) and *T. campestre* Schreber (hop trefoil) were also screened using this same method. As a consequence several isoflavones, e.g. daidzein, formononetin and their glycoside and glycoside malonate derivatives, were identified in these other three *Trifolium* species for the first time. Higher sensitivity of MS detection allowed the detection and quantification of very low levels of isoflavones in these other samples.

2. Experimental

2.1. Materials

Standard compounds, formononetin, genistein, glycitein, biochanin A and prunetin were purchased

from Indofine Chemical Company, Inc. (Somerville, NJ); pseudobabtigenin from Chromadex Inc.(Santa Ana, CA); daidzein from Sigma (St. Louis, MO); and calycosin, irilone and pratensein were purified from hydrolyzed extracts of red clover aerial part in this laboratory (Fig. 4). HPLC-grade methanol (MeOH), acetonitrile (ACN), ethanol (EtOH), aqueous ammonia, EtOAc and concentrated hydrochloric acid (HCl) were procured from Fisher Scientific Co. (Fair Lawn, NJ); formic acid was purchased from Acros Organics (NJ); and polyamide 6 was purchased from Sigma–Aldrich (St. Louis, MO). HPLC-grade water (18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA), and was used to prepare all solutions. Four batches of red clover samples were collected from fields in: New Brunswick, NJ; White Haven, PA; Catskills State Park, NY; and Shenandoah National Park, VA, in the summer of 2002, respectively; the three *Trifolium* species: *T. repense* L. (white clover); *T. hybridum* L. (alsike clover); and *T. campestre* Schreber (hop trefoil) were collected in New Brunswick, NJ. All botanical samples were identified in this laboratory.

2.2. Apparatus

HPLC separation was performed on a Phenomenex Prodigy ODS (3) column, 5 μm, 150 mm × 3.2 mm i.d. (Phenomenex Inc., Torrace, CA). For LC–ESI-MS and LC–MS–MS experiments, an Agilent 1100 Series LC–MSD trap (Agilent Technologies, Waldbronn, Germany) equipped with quaternary pump, photodiode array and multiple wavelength detector, thermostated column compartment, degasser, MSD trap with an electrospray ion source (ESI) and software of HP ChemStation, Bruker Daltonics 4.1 and DataAnalysis 4.1 was used. Waters Prep-HPLC with a Phenonex Luna phenyl–hexyl column, 10 μm, 250 mm × 30 mm i.d. (Phenomenex Inc., Torrace, CA), Delta 600 pump, 2487 Dual λ absorbance detector, 600 controller, 717 autosampler, fraction collector II, in-line degasser AF and software of Millennium 32 was used for standard purification.

2.3. Purification of standards of calycosin, irilone and pratensein from the aerial part of red clover

Red cloverin flowering was manually harvested (New Brunswick, NJ), air-dried, and then ground. Approximately 70 g of dried material was then refluxed in 700 ml 80% ethanol and 80 ml concentrated HCl for 2 h. The filtration was then evaporated under reduced pressure to remove ethanol; the pH of the solution was adjusted to 6 using aqueous ammonia, and then extracted with EtOAc. The EtOAc extract (3 g) was chromatographed on polyamide column using a step-gradient MeOH–H_2O (10–50% MeOH) and 250 ml fraction was collected. Those fractions containing the three target components as judged by LC–MS were collected and then further purified using prep-HPLC using the mobile phase of MeCN–H_2O (10% MeCN) at a flow rate of 7 ml/min to get calycosin (12 mg), irilone (25 mg) and pratensein (15 mg). The structures of these three compounds were then determined and verified by UV, MS and NMR spectrometric methods, and comparing with references as well [5,9,10,18,19].

2.4. Preparation of stock solutions, calibration standards and quality control (QC) samples

Individual stock solutions of 10 standards were prepared by dissolving the appropriate amounts of ∼5.0 mg in 15.0 ml of diluent (water and MeOH, 3:7). The final volume of each solution was then diluted to 25 ml with diluent. Calibration standards were prepared by diluting the stock solutions with diluent. The calibration curve ranges for UV and MS methods are presented in Table 3. In the calibration plots, 10 different concentrations were used for each analyte in triplicate by both UV and MS detector. QC samples were prepared separately by diluting the stock solutions with diluent. For UV and MS method, the concentrations of lower limit of quantitation (LLQ), low QC (LQC), middle QC (MQC) and high QC (HQC) are indicated in Tables 4 and 5.

2.5. Plant sample preparation

For qualitative study, ∼100 mg of finely ground material was extracted with 10 ml 80% methanol using sonication for 1 h at room temperature. The extracts were filtered through 0.45 μm filter and 20 μl extract was injected for each analysis [9]. The extraction procedure for quantitative analysis was modified from prior studies [13,15–17]. Approximately 500 mg of powdered red clover leaf, stem, root and flower, or

2000 mg of other three *Trifolium* species was placed into a 250 ml flask along with 50 ml of ethanol, 20 ml of DI water, and 8 ml of concentrated HCl. The mixture was refluxed for 2 h protected by N_2. The solution was filtered and diluted to volume of 100 ml. Each hydrolyzed sample of 5 μl filtered over 0.45 μm filter was analyzed by triplicate injections.

2.6. Liquid chromatographic and mass spectrometric conditions for identification of isoflavones

HPLC separation was performed with the mobile phase consisting of solvents A and B in gradient, where A was 0.1% formic acid (v/v) in water and B was 0.1% formic acid (v/v) in acetonitrile. The linear gradient profile was from 20 to 40% B in 40 min. The wavelength of UV detection was 254 nm. Column compartment was set at 25 °C. The flow rate was 1.0 ml/min and approximate 1/4 of its fluent was split into MSD trap. The electrospray ion mass spectrometer (ESI-MS) was operated under positive ion and auto MS–MS mode (threshold, 30,000) and optimized collision energy level of 100%, scanned from *m*/*z* 50 to 600. ESI was conducted using a needle voltage of 3.5 kV. High-purity nitrogen (99.999%) was used as dry gas and at a flow rate of 8 l/min, capillary temperature at 325 °C. Helium was used as nebulizer at 40 psi. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity. The auto MS–MS total ion chromatogram was processed by extracting the molecular ions of each isoflavone for identification.

2.7. Liquid chromatographic and mass spectrometric conditions for quantification of isoflavones

MS detection was conducted under collision energy level of 80% and scanned from *m*/*z* 100 to 320. Other MS parameter and LC conditions were the same as described above. Under multiple reaction monitoring (MRM) mode, protonated $[M^+ + H]$ ions were isolated for each isoflavone aglycone. The mass spectrometer was set into four time segments: (1) from 0 to 15 min for daidzein, glycitein and calycosin with isolation of *m*/*z* 255 and 285; (2) from 15 to 21 min for genistein and pratensein of *m*/*z* 271and 301; (3) from 21 to 27 min for pseudobabtigenin and formononetin of *m*/*z* 283 and 269; and (4) from 27 to 40 min for irilone, prunetin and biochanin A of *m*/*z* 285 and 299. The isolation width was set as *m*/*z* 1.0. The calibration curves were plotted using a 1/*x*-weighted quadratic model for the regression of peak area acquired from UV and MS detector versus analyte concentration. The concentrations of the QC and hydrolyzed plant samples were calculated from these linear equations.

3. Results and discussion

3.1. Identification of isoflavones in red clover and other Trifolium species

Simultaneous UV and auto MS–MS total ion chromatograms of 80% methanol extract of red clover leaf are shown in Fig. 2. The identities, retention time, protonated $[M + H]^+$ and characteristic fragment ions for individual peaks are presented in Table 1. The isoflavone aglycones, daidzein, formononetin, genistein, pseudobaptigenin, glycitein, calycosin, prunetin, biochanin A, irilone and pratensein, and the four isoflavone glucosides, daidzin, glycitin, genistin and ononin, were identified by comparison of the retention time and mass spectral data with those of standards.

Previous research has identified many isoflavone glycosides and glycoside malonates in red clover [5–10]. In general, the glucosyl group, and occasionally galactosyl group, is substituted on the 7/4′ position of aglycone and the malonyl group is linked to the 6″ position of sugar moiety. Based on their molecular ions and specific fragment ions of $[M^+ + H–malonyl]$ and $[M^+ + H–malonyl–glucosyl]$, most of the structures could be confirmed using this method. For example, peak 20 (t_R, 21.2 min) with molecular ion at *m*/*z* 533 and fragment ions of *m*/*z* 447 [533-malonyl] and 285 [533-malonyl–glucosyl] in MS spectrum (Fig. 3) was established as biochanin A-7-*O*-β-D-glucoside-6″-*O*-malonate. The structures of 10 isoflavone aglycones identified from red clover (Fig. 4) shows the proposed retro-Diels–Alder (RDA) fragment pathway in MS–MS spectra, in which precursor ion is the molecular ion of each aglycone. The glycosides or glycoside malonates of biochanin A, prunetin, glycitein and calycosin have the same molecular ion and characteristic fragment ions of $[M^+ + H–malonyl]$ and $[M^+ + H–malonyl–glucosyl]$ at *m*/*z* 533, 447 and 285, respectively. However, some

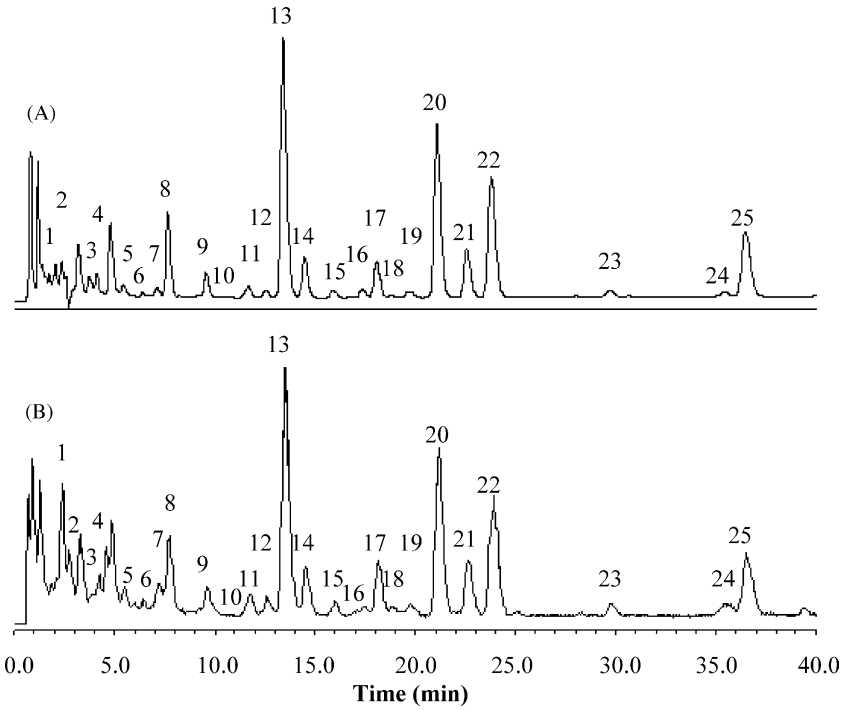


Fig. 2. Simultaneous (A) LC–UV and (B) auto MS–MS total ion chromatograms of red clover leaf extract. LC–MSD conditions were as described in Section 2. The identities, t_R-value and MS of each peak are listed in Table 1.

of these aglycones have different RDA fragment ions under MS–MS mode, *m/z* 137 for calycosin, *m/z* 153 for biochanin A (Fig. 5) and *m/z* 167 for prunetin and glycitein (precursor ion, *m/z* 285). Based on MS–MS data, the compounds derived from these four aglycones could be differentiated. Additionally, prunetin and glycitein derivatives have the same molecular ion and fragment ions including the aglycone RDA fragment at *m/z* 167. Prunetin can be differentiated as it has a 5-position hydroxyl group, which is easy

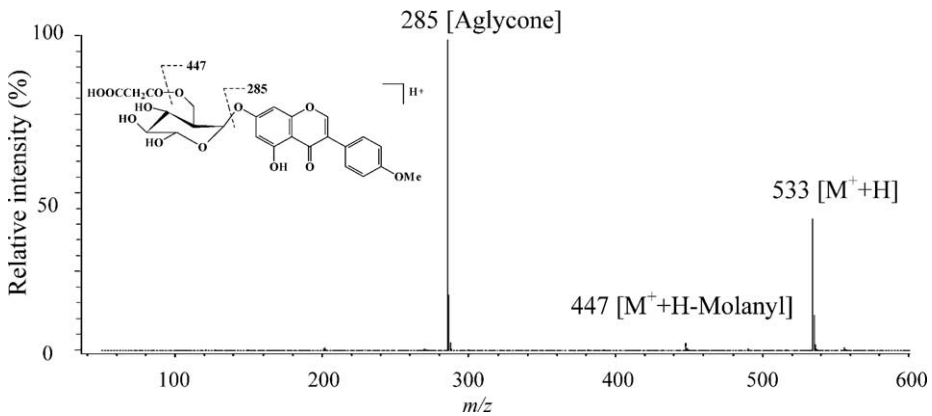


Fig. 3. MS spectrum of biochanin A–7-*O*-β-D-glucoside-6″-*O*-malonate (peak 20 in Fig. 2). Inset is the structure and its MS fragment pathway.

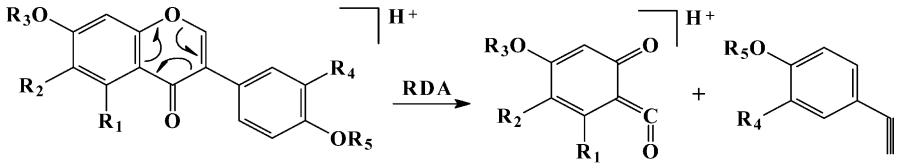
Table 1
Peak assignments for the analysis of red clover leaf extract

Peak	t_R (min)	$[M+1]^+$ (m/z)	MS fragment ion (m/z)	RDA fragment ion (m/z)	Identities	Compound code
1	2.0	417	255	[a]	Daidzein-G (Daidzin)[b]	1
2	2.7	447	285	[a]	Glycitein-G (Glycitin)[b]	2
3	3.8	433	271	153	Genistein-G (Genistin)[b]	3
4	4.2	503	255	137	Daidzein-G–M	4
5	5.5	533	285	137	Calycosin-G–M	5
6	6.5	549	301	[a]	Pratensein-G–M	6
7	7.1	445	283	137	Pseudobaptigenin-G	7
8a	7.7	431	269	137	Formononetin-G (Ononin)[b]	8
8b	7.7	519	271	[a]	Genistein-G–M	9
9a	9.6	549	463, 301	153	Pratensein-G–M	10
9b	9.6	255		[a]	Daidzein[b]	11
10	1.5	285		167	Glycitein[b]	12
11a	11.6	461	299	181	Irilone-G	13
11b	11.8	285		137	Calycosin[b]	14
12	12.6	531	283	137	Pseudobaptigenin-G–M	15
13	13.5	517	269	137	Formononetin-G–M	16
14a	14.3	517	269	137	Formononetin-G–M	17
14b	14.5	447	285	153	Biochanin A–G	18
15	15.9	447	285	167	Prunetin-G	19
16	17.5	271		153	Genistein[b]	20
17	18.2	547	299	181	Irilone-G–M	21
18	19.1	547	299	181	Irilone-G–M	22
19	19.8	547	299	181	Irilone-G–M	23
20	21.2	533	447, 285	153	Biochanin A–G–M	24
21a	22.6	533	447, 285	167	Prunetin-G–M	25
21b	22.8	283		[a]	Pseudobaptigenin[b]	26
22a	23.7	533	285	153	Biochanin A–G–M	27
22b	23.9	269		137	Formononetin[b]	28
23	29.8	299		181	Irilone[b]	29
24	35.5	285		167	Prunetin[b]	30
25	36.4	285		153	Biochanin A[b]	31

[a] Unable to measure.
[b] Identity based on MS spectral and retention data using authentic standards. In other cases, RDA fragment ion: MS–MS spectrum with precursor of aglycone molecular ion. G: glucosyl or galactosyl moiety; M: malonyl (in general, glucosyl group, occasionally galactosyl group was substituted on 7/4′ position of aglycone and malonyl group was linked to 6″ position of sugar moiety).

to combine with carbonyl group via hydrogen bond. As such, the polarity of prunetin and its derivatives are weaker than that of glycitein, and the retention time (t_R) on reverse phase column is longer. For example, the retention time of the prunetin glycoside (peak 15 in Fig. 2) is 15.9 min, while the glycitein glucoside (glycitin) is 2.7 min. Based on the above analysis, a total 31 isoflavones, including 9 aglycones, 8 glycosides and 14 glycoside malonates were identified in red clover leaf (Table 1). Comparative analysis of isoflavones by tissue showed that two major isoflavone aglycones, formononetin and biochanin A in stem, root and flowers are much lower than that found in the leaf (Fig. 6A–D). All 31 isoflavones are also detected in red clover stem, and 25 in root and 26 in flower as well (Table 2).

Previous chemical investigation on three *Trifolium* species of white clover, alsike clover and hop trefoil resulted in the identification of several saponins and phenolic components [14,20,21]. Based on available literature, no isoflavones had been detected in these plants. LC–MS comparative studies on four tissues of these three species led to the identification of 9, 11 and 4 isoflavones in white clover, alsike clover and hop trefoil, respectively (Table 2). The processed total ion chromatograms of white clover, alsike clover



Isoflavone **a+1**

Name	R_1	R_2	R_3	R_4	R_5	a+1	M^++H
Daidzein	H	H	H	H	H	137	255
Formononetin	H	H	H	H	Me	137	269
Genistein	OH	H	H	H	H	153	271
Pseudobaptigenin	H	H	H	-OCH$_2$-		137	283
Glycitein	H	OMe	H	H	H	167	285
Calycosin	H	H	H	OH	Me	137	285
Prunetin	OH	H	Me	H	H	167	285
Biochanin A	OH	H	H	H	Me	153	285
Irilone	OH	-OCH$_2$-		H	H	181	299
Pratensein	OH	H	H	OH	Me	153	301

Fig. 4. The structures of 10 isoflavone aglycones identified in red clover and their proposed MS–MS fragment pathway (RDA) with molecular ions as their precursors.

and hop trefoil leaf are shown in Fig. 6E–G. The results revealed that all these four species contain the isoflavones daidzein, formononetin and their glycosides and glycoside malonates (Table 2). Isoflavonoid isomers are commonly occurring in leguminous plants [9,22]. From red clover and other three related species, some isomers of glycoside malonates of pratensein, formononetin, irilone and biochanin A were detected in this investigation (Table 1). In addition, red clover was also reported to contain additional isoflavones of pectolinarigenin and afrormosin glucoside [9,10]. In this study with four sources of red clover materials collected from several regions, we did not find either of these two isoflavones at a detectable concentration.

3.2. Quantification of isoflavones in hydrolyzed red clover and other Trifolium species

Qualitative study revealed that red clover contained large mount of isoflavones, which are mainly available as malonate conjugates, e.g. glycoside malonates of formononetin, irilone and biochanin A (Fig. 2).

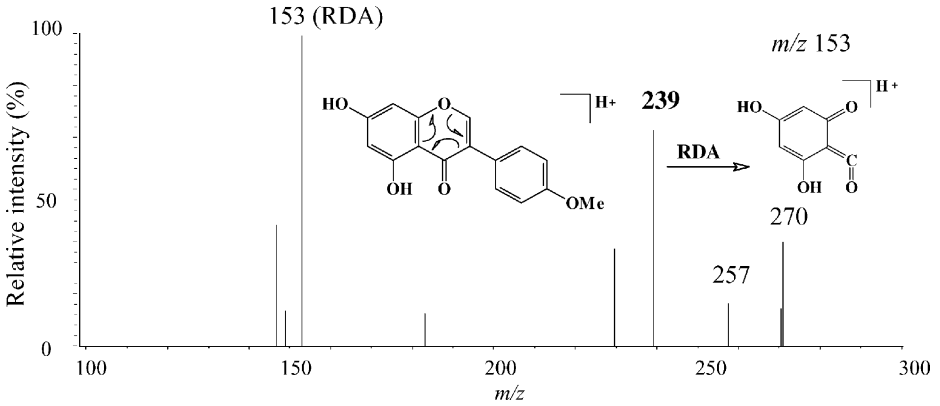


Fig. 5. MS–MS spectrum of biochanin A with precursor ion of *m/z* 285. Inset is the structure and its MS–MS fragment pathway. Ion peak at *m/z*: 153 is RDA fragment ion; 270 is $[M^+ + \text{H–Me}]$; 257 is $[M^+ + \text{H–CO}]$; and 239 may be $[257\text{-}H_2O]^+$.

Table 2
The presence of isoflavone in leaf, stem, root and flower of four *Trifolium* species

Compound code	Sample code															
	TP-L	TP-S	TP-R	TP-F	TR-L	TR-S	TR-R	TR-F	TH-L	TH-S	TH-R	TH-F	TC-L	TC-S	TC-R	TC-F
1	+	+	−	−	+	+	+	−	T	T	+	−	−	−	−	−
2	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
3	+	+	T	−	−	+	−	+	−	−	−	−	−	−	−	−
4	+	+	−	−	+	+	+	−	+	+	+	T	−	−	−	
5	+	+	+	T	−	−	−	−	−	−	−	−	−	−	−	−
6	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
7	+	+	+	T	−	−	−	−	+	+	+	−	−	−	−	−
8	+	+	+	+	+	+	T	+	+	+	+	+	T	T	T	T
9	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−
10	+	+	+	+	T	T	T	T	T	T	T	−	−	−	−	−
11	+	+	−	T	+	+	+	+	+	+	+	T	+	−	T	T
12	+	+	+	T	−	−	−	−	−	−	−	−	−	−	−	−
13	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
14	+	+	+	T	−	−	−	−	−	−	−	−	−	−	−	−
15	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	T
17	+	+	+	+	T	T	T	T	T	T	T	T	−	−	−	−
18	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
19	+	+	−	T	−	−	−	−	−	−	−	−	−	−	−	−
20	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−
21	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
22	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
23	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
24	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
25	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
26	+	+	+	T	−	−	−	−	+	+	T	+	−	−	−	−
27	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
28	+	+	+	+	+	+	T	+	+	+	+	+	T	T	T	−
29	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
30	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
31	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Total	31	31	25	26	8	9	8	9	11	11	11	8	4	3	4	3

Compound codes refer to Table 1. (+) Present; (−) not detectable; T: trace. TP: *T. pratense*; TR: *T. repense*; TH: *T. hybridum*; TC: *T. campestre*. L: Leaf; S: stem; R: root; F: flower. Total: total number of isoflavones identified.

The malonates are of biological interest in plants because this conjugated form can be utilized to store the less soluble isoflavone aglycones, and upon microbial infection, the aglycones are generated from the malonate conjugates [23,24]. Isoflavones are the common form of phytoestrogens. The glycosides and glycoside malonates in original plants are inactive. Clinical and preclinical studies found that the plant precursors, formononetin and biochanin A hydrolyzed from their glycosides and glycoside malonates are metabolized to daidzein and genistein (parent compounds) in humans and animals [1,2]. The parent compounds, daidzein and genistein may be absorbed or further metabolized to many specific metabolites including equol [1,2,25–30]. Thus, the presence of isoflavone aglycones may be directly related to the bioavailability. The major biotransformations in metabolism of isoflavones in humans and animals are illustrated in Fig. 1. Moreover, the malonate conjugates are not stable during processing [31–33] and it is very difficult to procure pure standards. Therefore, in this work, to facilitate the quantification and accurately evaluate the total isoflavones in red clover and its related species, the plant samples were hydrolyzed during

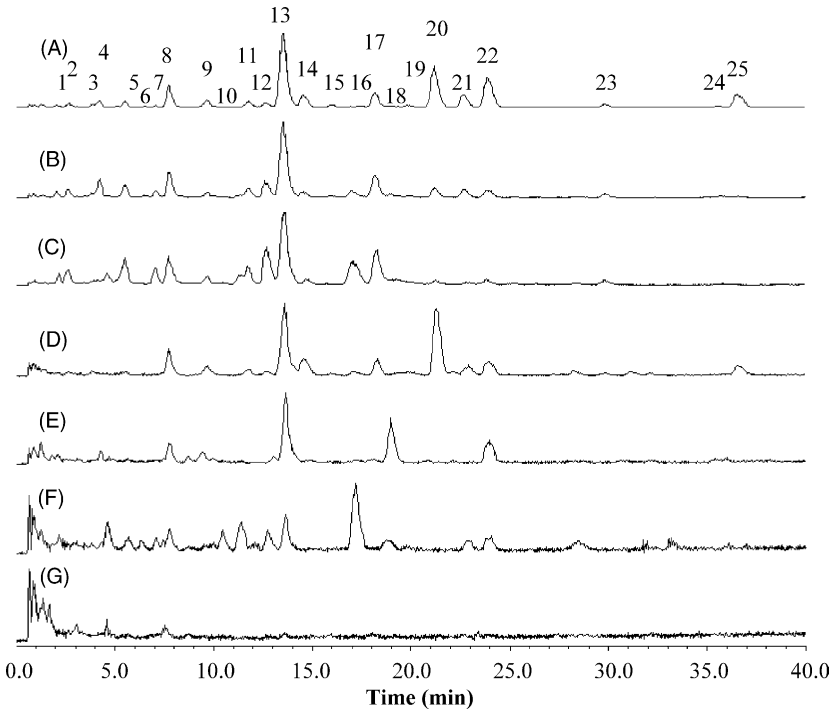


Fig. 6. Processed auto MS–MS total ion chromatograms of extracts of red clover: (A) leaf, (B) stem, (C) root, and (D) flower; and leaf of other three related species: (E) white clover, (F) alsike clover, and (G) hop trefoil. Reconstructed ion chromatograms were obtained by extracting the molecular ions of all isoflavones from their total ion chromatograms. LC–MSD conditions were as described in Section 2. Peak assignment in (A) red clover leaf is listed in Table 2. The presence of chemical profile for each sample is listed in Table 3.

extraction. Under optimized HPLC conditions, all 10 isoflavone aglycones, considered to be the major form of phytoestrogens, were successfully quantified in hydrolyzed red clover extract. As the isoflavones in the other *Trifolium* species of white clover, alsike clover and hop trefoil are very minor, MS detection with higher sensitivity was also applied to the analysis of the isoflavone aglycones in all four tissues of these

Table 3
Calibration curve ranges and regressions (r^2) of 10 analytes by UV and MS detection

Analyte	UV (ng/ml)	r^2	MS (ng/ml)	r^2
Daidzein	28.32–14500	1	7.08–3625	0.9957
Formononetin	32.23–16500	0.9999	8.06–4125	0.9972
Genistein	30.76–15750	0.9997	7.69–3937.5	0.9995
Pseudobaptigenin	21.97–11250	1	5.49–2812.5	0.9980
Glycitein	23.44–12000	1	5.86–3000	0.9999
Calycosin	25.39–1300	0.9995	6.35–3250	0.9964
Prunetin	25.39–1300	1	6.35–3250	0.9973
Biochanin A	25.39–1300	1	6.35–3250	0.9998
Irilone	23.44–12000	1	5.86–3000	0.9994
Pratensein	23.44–12000	1	5.86–3000	0.9997

Ten concentration levels were used for all calibration plots.

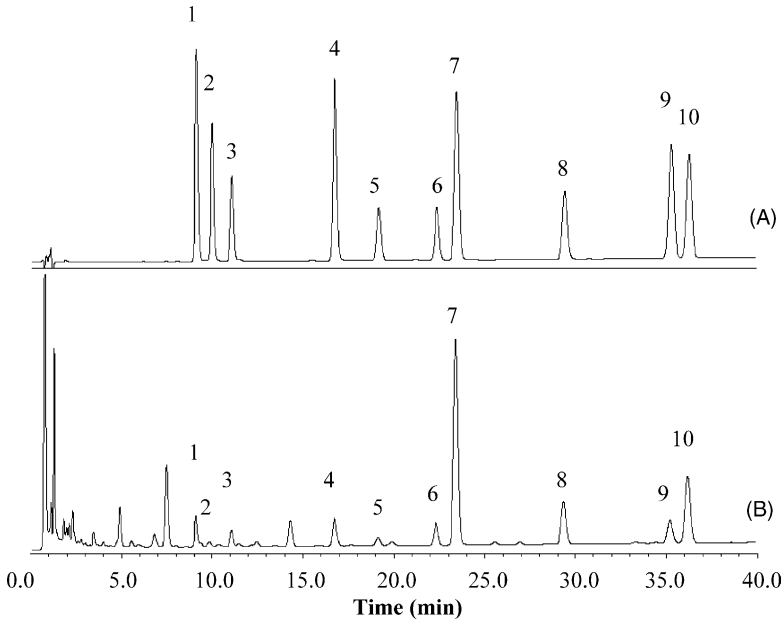


Fig. 7. LC–UV chromatograms of (A) a standard mixture and (B) hydrolyzed red clover leaf extract: (1) daidzein; (2) glycitein; (3) calycosin; (4) genistein; (5) pratensein; (6) pseudobaptigenin; (7) formononetin; (8) irilone; (9) prunetin; (10) biochanin A.

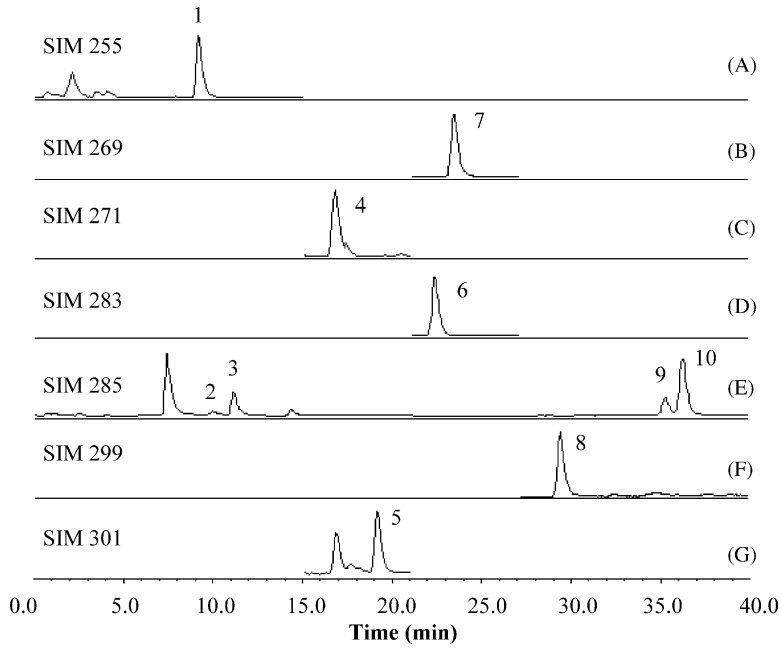


Fig. 8. Processed MS chromatograms of hydrolyzed red clover leaf extract. Reconstructed ion chromatogram for *m/z*: (A) 255, (B) 269, (C) 271, (D) 283, (E) 285, (F) 299, and (G) 301: (1) daidzein; (2) glycitein; (3) calycosin; (4) genistein; (5) pratensein; (6) pseudobaptigenin; (7) formononetin; (8) irilone; (9) prunetin; (10) biochanin A.

plants. For the plant extracts and spiked QC samples, all analytes were carried out in triplicate. The isoflavone concentrations in QC and plant samples were calculated from the regression equations.

3.2.1. HPLC method with UV detection

Using the conditions optimized under multiple preliminary assays, this system enables separation of 10 target isoflavone aglycones within 40 min. Fig. 7 shows chromatograms of a standard mixture and typical hydrolyzed red clover leaf extract under UV detection at wavelength of 254 nm. Peak assignments were made with single compound injections and MS spectral data. Baseline separation was successfully achieved for all 10 components.

The calibration was based on the triplicate analysis of each working solution at 10 concentration levels. The concentration ranges for the calibration curves of 10 standards were from ~24 to ~12,500 ng/ml, and regressions were >0.999, indicated in Table 3. The accuracy (deviation from nominal concentration) and precision (R.S.D.) of this method was assessed by analyzing the QC samples of LLQ, LQC, MQC, and HQC ($n = 10$). Results show that the assay accuracy and precision for the 10 analytes was within 15% (Table 4). The LLQs for the 10 analytes were

Table 4
Accuracy and precision of UV detection

	Analyte									
	1	2	3	4	5	6	7	8	9	10
LLQ (ng/ml)										
Nominal conc.	28.32	32.23	30.76	21.97	23.44	25.39	25.39	25.39	23.44	23.44
Mean conc.	27.53	35.98	30.61	25.46	24.55	28.27	27.71	27.48	23.70	25.72
S.D.	1.00	1.55	1.37	1.88	0.95	2.39	0.68	0.95	0.94	1.75
Accuracy (%)	−2.89	10.41	−0.50	13.70	4.50	10.18	8.36	7.59	1.10	8.86
Precision (%)	3.62	4.29	4.47	7.37	3.87	8.47	2.45	3.44	3.97	6.79
LQC (ng/ml)										
Nominal conc.	29.00	33.00	31.50	22.50	24.00	26.00	26.00	26.00	24.00	24.00
Mean conc.	28.72	36.72	31.74	26.10	24.67	28.18	26.30	26.22	21.61	26.76
S.D.	1.21	1.64	1.10	1.37	2.17	2.08	1.10	1.00	1.37	1.23
Accuracy (%)	−0.99	10.12	0.77	13.80	2.72	7.74	1.16	0.85	−11.08	10.32
Precision (%)	4.20	4.46	3.47	5.27	8.81	7.39	4.19	3.82	6.34	4.60
MQC2 (ng/ml)										
Nominal conc.	145	165	167.5	112.5	120	130	130	130	120	120
Mean conc.	146.84	172.92	160.39	126.59	116.13	139.94	131.84	135.29	124.14	121.22
S.D.	2.66	3.97	2.53	3.45	3.09	4.57	2.81	2.71	2.11	3.11
Accuracy (%)	1.25	4.58	−4.43	11.13	−3.34	7.10	1.40	3.91	3.33	1.01
Precision (%)	1.81	2.30	1.58	2.73	2.66	3.27	2.13	2.00	1.70	2.56
MQC1 (ng/ml)										
Nominal conc.	290	330	315	225	240	260	260	260	240	240
Mean conc.	300.36	342.22	321.69	246.47	252.97	269.53	262.98	263.12	245.92	253.92
S.D.	3.93	5.66	5.25	6.11	5.37	6.33	3.78	6.06	5.76	8.18
Accuracy (%)	3.45	3.57	2.08	8.71	5.13	3.54	1.13	1.19	2.41	5.48
Precision (%)	1.31	1.65	1.63	2.48	2.12	2.35	1.44	2.30	2.34	3.22
HQC (ng/ml)										
Nominal conc.	2900	3300	3150	2250	2400	2600	2600	2600	2400	2400
Mean conc.	2944.8	3387.8	3195.2	2347.5	2462	2624.5	2641.6	2640.1	2402.7	2437
S.D.	35.45	41.01	40.36	77.16	85.84	32.22	31.73	31.89	32.65	30.55
Accuracy (%)	1.52	2.59	1.41	4.15	2.52	0.93	1.58	1.52	0.11	1.52
Precision (%)	1.20	1.21	1.26	3.29	3.49	1.23	1.20	1.21	1.36	1.25

$n = 10$. (1) Daidzein; (2) glycitein; (3) calycosin; (4) genistein; (5) pratensein; (6) pseudobaptigenin; (7) formononetin; (8) irilone; (9) prunetin; (10) biochanin A.

established at ∼24 ng/ml, the lowest concentrations of analytes in the calibration curves (Table 4).

Using the method developed here, four sources of red clover samples collected from different regions in the USA were analyzed under UV detection. The contents of individual and total isoflavones in the four tissues of red clover were quantified (Table 6). The total isoflavones in the different plant sources ranged between 1.748 and 2.272% in leaf, 1.056 and 1.850% in stem, 1.360 and 2.853% in root, and with the lowest total isoflavone concentration found in the flowers (between 0.307 and 0.633%). The majority of isoflavones were formononetin, biochanin A and irilone.

3.2.2. HPLC method with MS detection

Compared to red clover, the isoflavones in the other *Trifolium* species were found in much lower concentrations, requiring an MS quantitative method to be developed. For MS detection, the analytes were set-up into four time segments. Under MRM mode, protonated $[M^+ + H]$ ion was isolated for individual target at *m*/*z* of compounds of: daidzein, 255; formononetin, 269; genistein, 271; pseudobabtigenin, 283; glycitein,

Table 5
Accuracy and precision of MS detection

	Analyte									
	1	2	3	4	5	6	7	8	9	10
LLQ (ng/ml)										
Nominal conc.	7.08	8.06	7.69	5.49	5.86	6.35	6.35	6.35	5.86	5.86
Mean conc.	7.43	8.38	7.63	5.96	5.16	5.91	6.13	6.49	5.63	6.30
S.D.	0.52	0.85	0.77	0.35	0.38	0.73	0.56	0.62	0.50	0.69
Accuracy (%)	4.67	3.77	−0.84	7.91	−13.51	−7.49	−3.60	2.18	−4.05	6.96
Precision (%)	7.03	10.10	10.04	5.94	7.41	12.41	9.12	9.52	8.88	10.97
LQC (ng/ml)										
Nominal conc.	14.50	16.50	15.75	11.25	12.00	13.00	13.00	13.00	12.00	12.00
Mean conc.	15.39	16.57	15.11	13.14	11.66	11.43	13.34	13.50	12.39	13.97
S.D.	1.42	1.49	1.11	0.62	1.34	0.88	1.70	1.07	0.85	1.12
Accuracy (%)	5.81	0.41	−4.25	14.36	−2.94	−13.73	2.56	3.71	3.13	14.09
Precision (%)	9.22	8.97	7.38	4.70	11.49	7.72	12.71	7.92	6.88	8.01
MQC2 (ng/ml)										
Nominal conc.	145.0	165.0	157.5	112.5	120.0	130.0	130.0	130.0	120.0	120.0
Mean conc.	148.81	148.93	146.25	110.89	111.01	119.12	135.27	122.76	122.72	116.95
S.D.	6.69	11.01	11.55	6.01	7.25	6.12	3.87	6.17	5.91	9.35
Accuracy (%)	2.56	−10.79	−7.69	−1.45	−8.10	−9.13	3.90	−5.90	2.21	−2.61
Precision (%)	4.49	7.39	7.89	5.42	6.53	5.14	2.86	5.03	4.82	7.99
MQC1 (ng/ml)										
Nominal conc.	290.0	330.0	315.0	225.0	240.0	260.0	260.0	260.0	240.0	240.0
Mean conc.	306.2	308.1	312.0	239.4	252.9	253.4	265.3	247.3	239.6	238.2
S.D.	15.30	20.10	18.59	24.13	9.62	11.56	8.89	10.16	6.04	7.46
Accuracy (%)	5.29	−7.11	−0.97	6.01	5.09	−2.59	2.00	−5.15	−0.18	−0.75
Precision (%)	5.00	6.52	5.96	10.08	3.80	4.56	3.35	4.11	2.52	3.13
HQC (ng/ml)										
Nominal conc.	1450	1650	1575	1125	1200	1300	1300	1300	1200	1200
Mean conc.	1497	1751	1671	1245	1265	1353	1347	1328	1210	1214
S.D.	73.11	17.74	54.48	27.43	33.69	32.31	32.75	37.74	16.21	17.78
Accuracy (%)	3.15	5.74	5.74	9.61	5.10	3.95	3.49	2.14	0.81	1.14
Precision (%)	4.88	1.01	3.26	2.20	2.66	2.39	2.43	2.84	1.34	1.46

$n = 10$. (1) Daidzein; (2) glycitein; (3) calycosin; (4) genistein; (5) pratensein; (6) pseudobaptigenin; (7) formononetin; (8) irilone; (9) prunetin; (10) biochanin A.

Table 6
Isoflavone contents in leaf, stem, root and flower of four *Trifolium* species (including four batch of red clover)

Sample code	Content (%)										
	1	2	3	4	5	6	7	8	9	10	Total
TP-NJ-L	0.035	0.673	0.066	0.034	0.043	0.058	0.061	0.541	0.149	0.097	1.756
TP-NJ-S	0.085	0.451	0.084	0.121	0.013	0.081	0.052	0.056	0.222	0.030	1.195
TP-NJ-R	0.012	0.325	0.076	0.293	0.062	0.206	0.019	0.021	0.288	0.067	1.369
TP-NJ-F	0.004	0.073	0.021	0.006	0.010	0.009	0.018	0.121	0.036	0.009	0.307
TP-VA-L	0.099	0.820	0.121	0.056	0.061	0.053	0.151	0.532	0.282	0.097	2.272
TP-VA-S	0.094	0.729	0.073	0.252	0.029	0.126	0.049	0.169	0.235	0.095	1.850
TP-VA-R	0.021	0.319	0.060	0.289	0.054	0.247	0.027	0.017	0.231	0.096	1.360
TP-PA-L	0.040	0.430	0.069	0.028	0.074	0.029	0.091	0.737	0.172	0.077	1.748
TP-PA-S	0.051	0.219	0.056	0.112	0.009	0.056	0.027	0.038	0.150	0.022	0.740
TP-PA-R	0.014	0.593	0.089	0.580	0.050	0.585	0.025	0.074	0.214	0.214	2.438
TP-NY-L	0.029	0.459	0.126	0.547	0.063	0.372	0.023	0.020	0.253	0.133	2.024
TP-NY-S	0.042	0.432	0.054	0.062	0.010	0.077	0.056	0.101	0.133	0.091	1.056
TP-NY-R	0.044	0.863	0.184	0.100	0.034	0.069	0.096	0.907	0.216	0.339	2.853
TP-NY-F	0.005	0.129	0.039	0.013	0.006	0.018	0.038	0.167	0.052	0.166	0.633
TR-L	0.0005	0.0241	0.0082	–	–	–	–	–	–	–	0.0327
TR-S	0.0004	0.0282	0.0069	–	–	–	–	–	–	–	0.0354
TR-R	0.0006	0.0150	0.0055	–	–	0.0042	–	–	–	–	0.0253
TR-F	0.0001	0.0040	0.0171	–	–	–	–	–	–	–	0.0213
TH-L	0.0006	0.0110	–	0.0164	0.0103	0.0040	–	–	–	–	0.0423
TH-S	0.0003	0.0050	–	0.0075	0.0103	0.0101	–	–	–	–	0.0333
TH-R	0.0005	0.0129	0.0054	0.0050	0.0098	0.0094	–	–	–	–	0.0431
TH-F	0.0002	0.0054	–	0.0014	–	–	–	–	–	–	0.0070
TC-L	0.00004	0.00024	–	–	–	–	–	–	–	–	0.00028
TC-S	0.00004	0.00057	–	–	–	–	–	–	–	–	0.00061

TP: *T. pratense*; TR: *T. repense*; TH: *T. hybridum*; TC: *T. campestre*. NJ: New Jersey, New Brunswick; PA: Pennsylvania, White Haven; VA: Virginia, Shenandoah National Park; NY: New York, Catskills State Park. L: Leaf; S: stem; R: root; F: flower; (–) not detectable. Isoflavone content of *T. pratense* calculated using UV detection and isoflavone content of *T. repense*, *T. hybridum* and *T. campestre* calculated using MS detection. (1) Daidzein; (2) glycitein; (3) calycosin; (4) genistein; (5) pratensein; (6) pseudobaptigenin; (7) formononetin; (8) irilone; (9) prunetin; (10) biochanin A.

calycosin, prunetin and biochanin A, 285; irilone, 299; and pratensein, 301. Fig. 8 illustrates the MS chromatogram of hydrolyzed red clover leaf extract with selected ion monitoring (SIM) that demonstrates baseline separation of the 10 components in complex plant metrices within 40 min.

The calibration curves were constructed by injecting working solutions between ∼6 and ∼3125 ng/ml at 10 concentration levels in triplicate, and regression values were >0.99 (Table 3). The LLQ is ∼6 ng/ml and LLQs for individual compounds are presented in Table 5. The accuracy and precision of MS method was also confirmed by analyzing the QC samples of LLQ, LQC, MQC, and HQC ($n = 10$). The accuracy and precision for all 10 standards were found to be <15% (Table 5). The sensitivity of this MS method is significantly higher than that of UV detection, and the results are in good agreement with the minor isoflavone content in white clover, alsike clover and hop trefoil. The individual and total isoflavones among the four tissues of these three species also significantly varied (Table 6). The total isoflavones in different parts of the other *Trifolium* species ranged from 0.0213 to 0.0354% in white clover, 0.0070 to 0.0431% in alsike clover and 0.00028 to 0.00061% in hop trefoil.

4. Conclusions

High-performance liquid chromatography combined with ultraviolet and electrospray ionization-mass spectrometric detector (HPLC–UV–ESI-MSD) has been applied to the study of isoflavones in plant

tissues of four *Trifolium* species. Under the multiple optimized HPLC and MSD conditions, 31 isoflavones including 9 isoflavone aglycones, 8 alycosides and 14 glycoside malonates were identified from red clover according to their molecular ions and specific fragment ions under MS and MS–MS mode, and in comparison with the standard isoflavones as well. In addition, several of these isoflavones of daidzein, formononetin and their glycosides and glycoside malonates have for the first time also been detected in white clover, alsike clover and hop trefoil.

A simple, reliable, and sensitive method has been developed for quantitation of total isoflavones in hydrolyzed plant extracts by HPLC with UV and MS detector. Using this method, all 10 isoflavone aglycones were totally separated and eluted individually within 40 min. This is the first method to simultaneously quantify all 10 isoflavones in red clover. A more sensitive MS method was developed that also allowed the analysis of the isoflavones found only in minor amounts in white clover, alsike clover and hop trefoil. Validation of this quantitative method led to the LLQ at ∼24 ng/ml for UV detection and ∼6 ng/ml for MS detection, respectively. The accuracy and precision (R.S.D.) for both UV and MS method were well below 15% at the concentration of LLQ.

For this study, several standards such as irilone, calycosin and pratensein were not commercial available. Using polyamide chromatography and Prep-HPLC, we have purified these three standards from hydrolyzed red clover extracts and elucidated their structures by UV, MS, MS–MS and NMR spectrometric methods.

Using the method described, four tissues from four sources of red clover samples collected from regions in the USA, as well as from three additional *Trifolium* species, white clover, alsike clover and hop trefoil, were determined. The results revealed that the isoflavone profile and content between different species showed great variation between species and within species by tissue. The isoflavone content in red clover is much higher than that found in the other related species, and total isoflavones in red clover are significantly higher in the leaf, stem, and root than in the flower.

This newly developed LC–MSD method is routinely manageable and allows successful qualification and quantitation of all isoflavones in both original and hydrolyzed red clover samples, and related species. This study should provide a framework and new improved method for quality control of red clover products.

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