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Short communication

Analysis of flavonoids from red clover by liquid chromatography–electrospray mass spectrometry

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Abstract

A high-performance liquid chromatographic method paired with photodiode-array and electrospray mass spectrometer detectors enables us to rapidly characterize the flavonoid constituents from red clover. A total of eleven flavonoids were identified. Among them, seven flavonoids – genistin, isoquercitrin, ononin, daidzein, sissotrin, formononetin and biochanin A – were unambiguously identified based on their abundant $[M+H]^+$, $[M+Na]^+$ ions, UV spectra, retention time and analysis of hydrolyzed products. The purity of three peaks, for pratensein, pectolinarigenin and pseudobaptigenin, is not enough to have predominant $[M+H]^+$ or $[M+Na]^+$ ions, but their existence is believable. Hyperoside has intense $[M+H]^+$ and $[M+Na]^+$ ions. Its identification is still tentative, because there are several other quercetin glycosides which will have the same $[M+H]^+$ ion.

Keywords: Trifolium pratense; Flavonoids

1. Introduction

Standardization of botanical extracts have become increasingly important for the food industry and for research of herbal medicine. They require a clear chemical characterization of the botanical extract. Comparison of retention time with reference compounds sometimes is not enough to identify chromatographic peaks. High-performance liquid chromatography (HPLC) with on-line recorded UV spectra and mass spectra can provide a powerful tool to confirm peak identification.

There are different types of interfaces to couple HPLC and mass spectrometry (MS) in order to accomplish nebulization and vaporization of liquid, ionization of sample, removal of the excess solvent, and transfer of the ions into the mass analyzer. The thermospray (TS) interface has been most widely applied in phytochemical analysis [1]. Electrospray (ES) interface has become commercially available only recently. Unlike TS, ES operates without the input of heat into the spray ionization step, so that labile and polar samples are ionized without thermal degradation. It is very suitable for the analysis of bio-macromolecules.

In 1988, Fenn et al. first reported ES-MS spectra of multiply protonated molecular ions of proteins having 45 positive charges [2]. Since then, ES-MS coupled only with capillary electrophoresis (CE) has been successfully used for analysis of amino acids, catecholamides and various polypeptides [3]. There are only a few papers published dealing with the

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application of HPLC–ES-MS. Bruins et al. separated five synthetic monosulfonated azo dyes and measured their $[M-H]^-$ ions of the free acid form by HPLC–ES-MS [4]. Bleicher and Bayer analyzed oligonucleotides using HPLC–ES-MS [5].

The aim of this investigation was to study the applicability of HPLC–ES-MS for identification and purity analysis of flavonoid constituents of red clover extract.

Red clover, *Trifolium pratense* L. (family Leguminosae), is a major forage plant. Extract of red clover is used as a flavor ingredient in many food products. Its flowerhead also has been used medicinally as a dermatological agent, mild antispasmodic and expectorant. Its isoflavone constituents have estrogenic properties [6]. Table 1 lists the sixteen flavonoids discussed in this report.

Formononetin (**5**) and biochanin A (**7**) were reported to be two major isoflavonoids (around 0.1–0.9% in dry forage) [7]. Minor flavonoids (below 0.08% in dry forage) are genistin (**4**), genistein (**3**), daidzin (**2**), daidzein (**1**), pratensein (**12**), pectalinarigenin (**11**), calycosin (**10**), trifoside (**9**), pseudobaptigenin (**13**) and isoquercitrin (**15**) [8–11]. So far, there are no reports identifying all peaks in the HPLC chromatogram of red clover.

2. Experimental

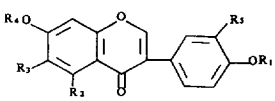
2.1. Instrumentation

HP 1090 Series II HPLC [Hewlett-Packard, Palo Alto, CA, USA] with a photodiode-array detector set at 260 nm was coupled with HP 5989 B quadrupole mass spectrometer.

UV spectra were taken at a region at 200–500 nm. Chromatographic conditions were: column, HP ODS

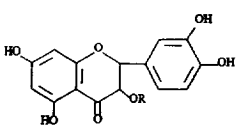
Table 1
Structure of isoflavonoids and flavonols in red clover

Isoflavonoids



No.	Compound	R_1	R_2	R_3	R_4	R_5	M_r
1	Daidzein	H	H	H	H	H	254
2	Daidzin	H	H	H	Glucose	H	416
3	Genistein	H	H	OH	H	H	270
4	Genistin	H	H	OH	Glucose	H	432
5	Formononetin	CH_3	H	H	H	H	268
6	Ononin	CH_3	H	H	Glucose	H	430
7	Biochanin A	CH_3	H	OH	H	H	284
8	Sissotrin	CH_3	H	OH	Glucose	H	446
9	Trifoside	Glucose	H	H	CH_3	H	374
10	Calycosin	CH_3	H	H	H	OH	284
11	Pectolinarigenin	CH_3	OH	OCH_3	H	H	314
12	Pratensein	CH_3	OH	H	H	OH	300
13	Pseudobaptigenin	$-CH_2-$	H	H	H	-O-	282

Flavonols



	Compound	R	M_r
14	Quercetin	H	302
15	Isoquercitrin	Glucose	464
16	Hyperoside	Galactose	464

Hypersil, 5 μm, 200×2.1 mm; eluent: (A) water (0.25% HOAc), (B) MeOH; linear gradient elution, 0–100% B at 30 min; flow-rate, 0.2 ml/min; temperature, 45°C.

Mass range measured: 150–600 u; quadrupole temperature: 150°C; EM volts 2010. The spectra were acquired in the positive mode.

ES interphase was HP 59987 A; drying N_2 temperature, 350°C, 40 ml/minute; nebulizing N_2, $5.5 \cdot 10^5$ Pa (80 psi).

2.2. Plant material and sample preparation

Red clover flower was purchased from Trout Lake Farm in Washington state, USA.

A 5-g mass of the dried flower of red clover was refluxed with 100 ml of 70% ethanol for 1 h. The ethanol solution was filtered through a filter paper (Whatman, No. 6). A 1-ml aliquot was diluted to 5 ml with MeOH and filtered through a 0.45-μm nylon acrodisk 13 filter (Gelman, USA) for HPLC analysis. A 10-μl volume of the sample solution was injected onto the HPLC column.

Hydrolysis of sample: 20 ml 2 *M* HCL was added to 10 ml of the above sample solution and refluxed for 3 h. A 10-μl volume of the hydrolyzed solution was filtered through a 0.45-μm nylon acrodisk 13 filter for HPLC analysis.

2.3. Standard compounds and chemicals

Daidzin (**2**) and daidzein (**1**) were purchased from Extrasynthese, France. Genistin (**4**), genistein (**3**), quercetin (**14**) and biochanin A (**7**) were purchased from Sigma, USA. Isoquercitrin (**15**) was purchased from Indofine, USA. MeOH is HPLC grade (VWR, USA).

3. Results and discussion

3.1. HPLC–MS of flavonoid standard compounds

Seven standard flavonoids – daidzin (**2**), genistin (**4**), isoquercitrin (**15**), daidzein (**1**), quercetin (**14**), genistein (**3**) and biochanin A (**7**) – were chromatographed in order to determine their retention times, UV spectra and mass spectra for comparison with the chromatogram of extract of red clover. Their HPLC and total ion chromatograms are shown in Fig. 1. Their retention time (t_R), $[M+H]^+$, $[M+Na]^+$ and UV λ_{max} values are shown in Table 2.

3.2. HPLC–MS of extract of red clover flower

HPLC and total ion chromatograms of 70% ethanol extract of red clover flower are shown in Fig. 2. The retention time, $[M+H]^+$, $[M+Na]^+$ and UV λ_{max} values and their identifications for individual peaks are listed in Table 3. Genistin (**4**), isoquercitrin (**15**), ononin (**6**), daidzein (**1**), sissotrin (**8**), formononetin (**5**) and biochanin A (**7**) were unambiguously identified based on their intense molecular ions $[M+H]^+$, adduct ions $[M+Na]^+$, UV spectra, retention time and analysis of hydrolyzed products. Pratensein (**12**), pectolinarigenin (**11**) and pseudobaptigenin (**13**) have no predominant $[M+H]^+$ or $[M+Na]^+$ ions, because their peaks are coeluted with other compounds. Their identification is tentative, but their existence in red clover is believable.

3.3. The identification of flavonoid glycosides

The HPLC chromatogram of the hydrolyzed sample (not shown in this report) shows that the heights of peaks 3 (isoquercitrin), 4 (ononin) and 6 (sisotrin) are significantly decreased, and the heights of peaks 10 (formononetin) and 11 (biochanin A) are largely increased. Between peaks 4 and 6, there appears a new peak; its retention time and UV spectrum are the same as that of quercetin (**14**). This fact confirms that peaks 3, 4 and 6 should be glycosides of quercetin (**14**), formononetin (**5**) and biochanin A (**7**), respectively.

Moreover, the mass spectra of both peaks 2 and 3 show not only an intense molecular ion $[M+H]^+$ at m/z 465 and an adduct ion $[M+Na]^+$ at m/z 487, but also an intense molecular ion $[M+H]^+$ at m/z 303 which is a protonated molecule $[464-162+H]^+$ of quercetin (**14**). The retention time and UV spectra of peaks 2 and 3 are the same as those of hyperoside (**16**) and isoquercitrin (**15**), respectively. Since there is no report in the literature that hyperoside (**16**) exists in red clover, and there are several glycosides of quercetin (**14**) which have the same molecular

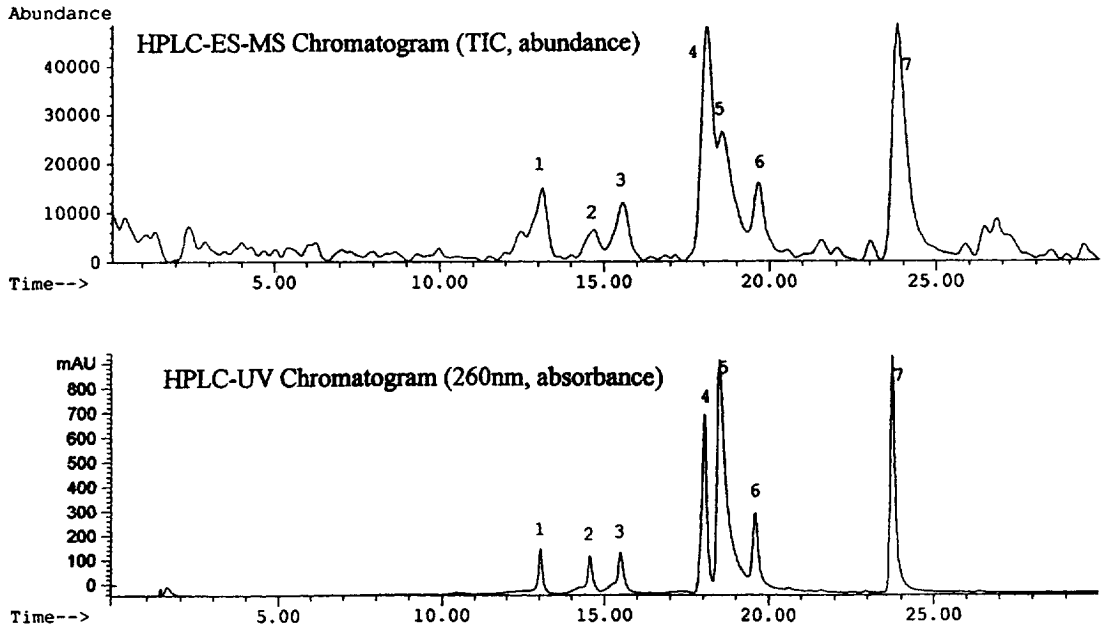


Fig. 1. Simultaneous HPLC-UV and HPLC–ES-MS chromatogram of flavonoid standard compounds, without post-column stream splitting. Chromatographic conditions were as described in Section 2. The following compounds are indicated: 1=daidzin (**1**); 2=genistin (**4**); 3=isoquercitrin (**15**); 4=daidzein (**1**); 5=quercetin (**14**); 6=genistein (**3**); 7=biochanin A (**7**).

mass, 464, the identification of hyperoside (**16**) is still tentative.

The mass spectra of peaks 4 and 6 also show intense molecular ions $[M+H]^+$ at *m/z* 269 and $[M+H]^+$ at *m/z* 285 respectively, providing further evidence that they are the glycosides of formononetin (**5**) and biochanin A (**7**), respectively, also called ononin (formononetin-7-glucoside) (**6**) and sissotrin (biochanin A-7-glucoside) (**8**). Ononin (**6**) was first discovered in the plant *Ononis spinosa* [12]. Literature has not yet reported its existence in red clover. The mass spectra of peaks 2, 3, 4 and 6 are shown in Fig. 3.

ES-MS is a "soft" ionization process. The compounds of interest exhibited only molecular ions in spectrum, and no fragment ions were observed. The amount of Na adduct will depend on the quality of the water, solvent and other conditions but forming $[M+Na]^+$ ion may confirm the molecular mass. The flavonoid glycosides probably are partially ionized in

Table 2
The values of t_R, $[M+H]^+$, $[M+Na]^+$, UV λ_{max} of flavonoids standard compounds

Peak number	Compound	t_R (min)	$[M+H]^+$ *m/z*	$[M+Na]^+$ *m/z*	UV λ_{max} (mn)
1	Daidzin	13.0	417	439	256, 313 sh
2	Genistin	14.6	433	455	261, 330 sh
3	Isoquercitrin	15.6	465	487	255, 365
4	Daidzein	18.1	255	277	249, 259 sh, 303 sh
5	Quercetin	18.6	303	325	255, 370
6	Genistein	19.7	271	293	261, 328 sh
7	Biochanin A	23.7	285	307	261, 330 sh

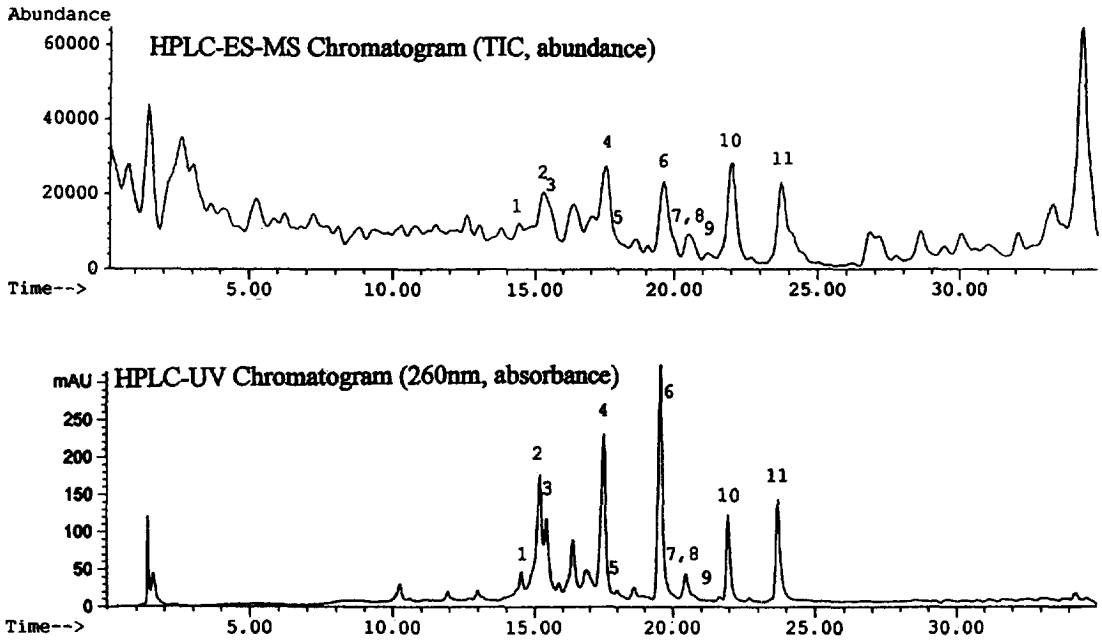


Fig. 2. Simultaneous HPLC–UV and HPLC–ES-MS chromatogram of 70% ethanol extract of red clover, without post column stream splitting. Chromatographic conditions were as described in Section 2. The following compounds are indicated: 1=genistin (**4**); 2=hyperoside (**16**); 3=isoquercitrin (**15**); 4=ononin (**6**); 5=daidzein (**1**); 6=sissotrin (**8**); 7=pratensein (**12**); 8=pectolinarigenin (**11**); 9=pseudobaptigenin (**13**); 10=formononetin (**5**); 11=biochanin A (**7**).

ES conditions to eliminate a hexosyl moiety and become an aglycone before entering the mass spectrometer. Therefore, their molecular ions $[M-162+H]^+$ of aglycone are also abundant, along with their molecular ions of glycosides.

Our results showed that HPLC–MS is a powerful

Table 3
Peak assignments for analysis of red clover extract

Peak number	t_R (min)	$[M+H]^+$ *m/z*	$[M+Na]^+$ *m/z*	UV λ_{max} (nm)	Identification
1	14.6	433	455	261, 330 sh	Genistin
2	15.2	465	487	257, 362	Hyperoside
3	15.4	465	487	255, 365	Isoquercitrin
4	17.7	430	453	258, 301 sh	Ononin
5	18.0	255	277	[a]	Daidzein
6	19.6	447	469	255, 325 sh	Sissotrin
7	20.5	301	323	[a]	Pratensein[b]
8	20.5	315	337	[a]	Pectolinarigenin[b]
9	21.2	283	305	[a]	Pseudobaptigenin[b]
10	22.0	269	291	248, 311	Formononetin
11	23.8	285	307	261, 330 sh	Biochanin A

[a] Not able to record.
[b] Peak is not pure; $[M+H]^+$ or $[M+Na]^+$ is not predominant.

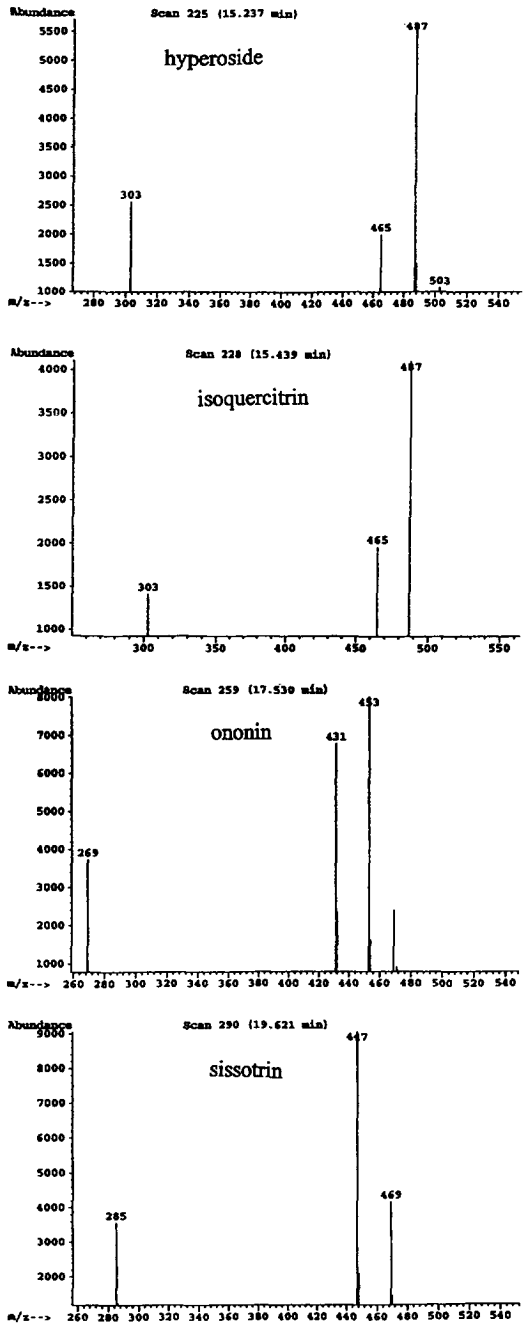


Fig. 3. Mass spectra of hyperoside, isoquercitrin, ononin and sissotrin.

tool for rapid and reliable peak identification with small amounts of plant material. Therefore, it will help to localize unknown compounds for further isolation. Knowing the purities of the peaks, it also will make quantitative analysis more accurate.

Acknowledgments

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