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Identification of isoflavones and their conjugates in red clover by liquid chromatography coupled with DAD and MS detectors

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Ultra performance liquid chromatography (UPLC) coupled with a diode array detector (DAD) and mass spectrometry (MS) was applied for identification of isoflavones and their conjugates in red clover (*Trifolium pratense* L.) growing in Lithuania. Isoflavones were extracted with methanol/water (9:1, *v/v*) using sonication for 60 min at room temperature. Optimized separations were carried out on the Acquity UPLC BEH C18 column in the gradient elution mode using the mobile phase composed of water and methanol containing 0.25% (*v/v*) acetic acid. Based on UV spectra, MS data, elution order and elution profiles, measured before and after acid hydrolysis, a total of 16 isoflavone aglycones, their glucosides and glucoside malonates were identified in red clover extract.

Key words: red clover, isoflavones, conjugates, identification, UPLC-DAD-MS

INTRODUCTION

Flavonoids are the most widely spread and diverse group of polyphenols, which belong to a large family of secondary plant metabolites [1]. They have the general structure of a 15-carbon skeleton (Fig. 1A), which consists of two phenyl rings (A and B) and heterocyclic ring (C). Flavonoids act as UV protectants in plant cells and as pigment sources for flower coloring compounds [2]. In human health, flavonoids have received considerable attention because of their protective role against cancer and heart diseases [3]. Because of the importance of flavonoids, the identification, structural determination and quantitation of these compounds have become a hot topic in food and plant sciences.

Red clover (*Trifolium pretense* L.) has recently received considerable interest as the richest source of isoflavones, a structurally special subclass of flavonoids with the B-ring linked to the *C*-3 position of the C-ring (Fig. 1B). The isoflavone concentration in red clover has been reported to be 2–10 times higher than in soybean seeds, the more common source of isoflavones [4]. Extracts of red clover are becoming increasingly popular for the treatment of menopausal symptoms [5, 6]. Furthermore, the plant is used to treat whooping cough, asthma, skin complains, eye diseases, and external cancers [7–9].

High-performance liquid chromatography (HPLC) coupled with UV/Vis or mass spectrometric (MS) detectors is the most common technique currently employed for the identification/determination of flavonoids in red clover [4, 10–15]. Like most flavonoids, isoflavones can exist in plants as free aglycones and/or as their conjugates. If the OH group on the *C*-7 position reacts with one glucose molecule (Fig. 1C), it will become a 7-O-isoflavone glucoside [1]. This is the most common glucoside form of isoflavones which

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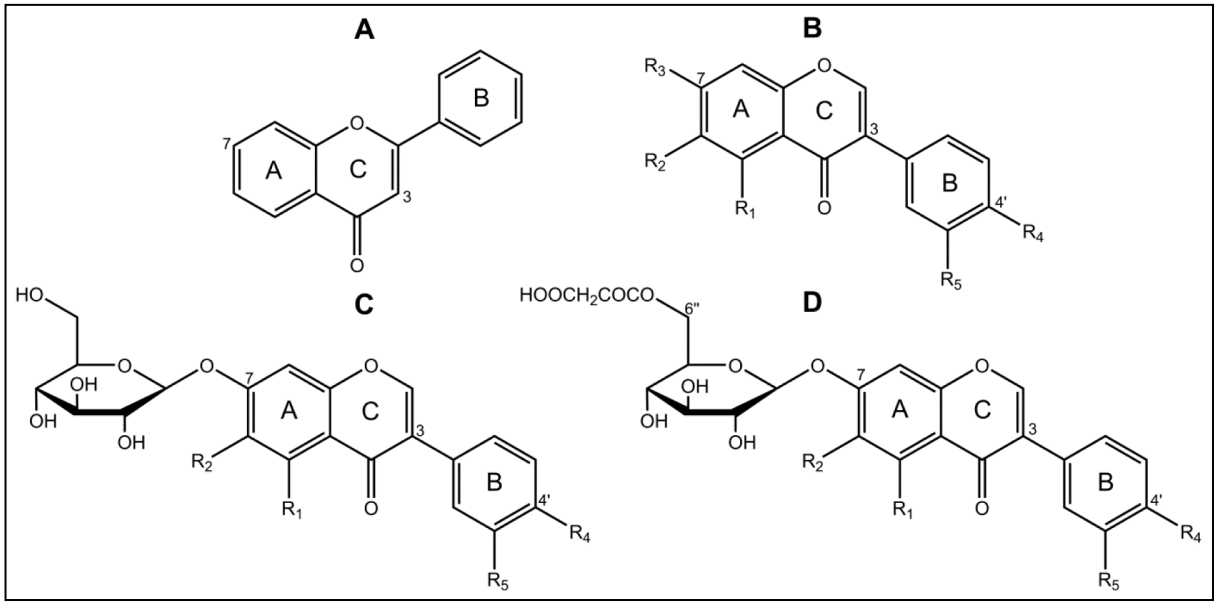

Fig. 1. General structures of compounds studied. A – general structure of flavonoid; B – isoflavone aglycone; C – isoflavone-7-*O*-glucoside; D – isoflavone-7-*O*-glucoside-6″-malonate

exists naturally in plants. Furthermore, acylation of the glucosides, in which one or more of the sugar hydroxyl groups are derivatized with an acid such as malonic or less often acetic is also observed (Fig. 1D) [16]. Forms of isoflavones are important because they directly affect the bioavailability and thus the bioactivity of isoflavones. However, in most cases plant materials are analyzed by hydrolysis of the conjugated forms and subsequent determination of the released free aglycones. In a few previous studies several isoflavone aglycones and their glucoside and glucoside malonate derivatives have been identified in red clover by using HPLC-UV and/or HPLC-MS techniques [17–19]. The distribution of these compounds, however, is dependent on many factors, including cultivar, environment, climate, etc.

In the present study, ultra performance liquid chromatography (UPLC) coupled with a diode array detector (DAD) and quadrupole time-of-flight mass spectrometry (q-TOF-MS) was applied for identification of isoflavones and their conjugates in red clover growing in Lithuania.

EXPERIMENTAL

Separations were performed on a Waters Acquity UPLC system equipped with an Acquity UPLC DAD detector and connected to a MicrOTOF-QII quadrupole time-of-flight mass spectrometer (Bruker). Acquity UPLC BEH C18 (100 mm × 2.1 mm I. D., 1.7 μm, Waters) column was used in the experiments. The mobile phase was composed of (A) water and (B) methanol/water (80:20 *v/v*) both containing 0.25% (*v/v*) acetic acid. The gradient elution program was as follows: 0–15 min, 2–100% B linear; 15–17 min, 100–2% B linear; 17–22 min, 2% B isocratic. The column temperature was maintained at 30 °C. The mobile phase flow rate was 0.25 mL/min. The injection volume was 2 μL using a partial loop with needle overfill injection mode. DAD spectra were recorded between 200 and 500 nm. The eluent was then directed to the mass spectrometer equipped with an electrospray ionization (ESI) source operating in a negative ion mode. The values of MS parameters were the following: mass range from 100 to 1 000 *m/z*; capillary voltage 4 kV; drying gas temperature 180 °C; drying gas flow 8 L/min; nebulizing gas pressure of 0.6 bar. All of the spectra were calibrated prior to compound identification by injecting the calibrant at the beginning of each UPLC run. The accurate mass data were processed using the software DataAnalysis 4.0 (Bruker).

The red clover samples were collected at Kėdainiai, central Lithuania in the summer of 2014. Fresh samples were chopped, fixed at 105 °C for 20 min, dried at 65 ± 5 °C and ground in a cyclonic mill with a 1 mm sieve. The extraction was performed according to a slightly modified procedure described by Lin et al. [20]. Briefly, the representative amount of sample (0.100 g) was extracted with 10 mL of methanol/water (9:1, *v/v*) using sonication for 60 min at room temperature. The extract was then filtered through 0.2 μm nylon syringe filter and analyzed immediately after extraction.

Hydrolyzed samples were prepared as follows: 2 mL of extract solution was mixed with 0.4 mL of 37% HCl and heated in a sealed vial at 80–85 °C for 1.5 h. The solution was then filtered and analyzed.

RESULTS AND DISCUSSION

In order to optimize separation conditions, the chromatographic profiles of representative red clover extract were measured under reversed phase UPLC conditions with different mobile phase compositions. Several binary solvent

systems of acetonitrile–water and methanol–water with or without acetic acid were tested. Our preliminary studies indicated that a mobile phase comprising a mixture of methanol–water acidified with 0.25% (*v/v*) acetic acid provided the best separation of isoflavones and their conjugates. The acidification of the mobile phase reduced the peak tailing for some eluted compounds and also enhanced the ESI-MS signal response. The optimized gradient elution conditions are described in the Experimental section.

Figure 2 shows the UPLC-DAD chromatogram of a red clover extract obtained under optimized elution conditions at 260 nm. It can be seen that all of the compounds were eluted within 15 min and most of them were completely resolved.

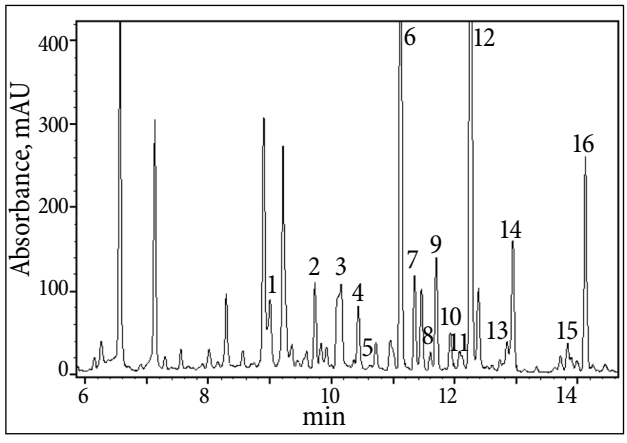

Fig. 2. UPLC-DAD chromatogram of a red clover extract obtained under optimized elution conditions at 260 nm

Isoflavones and their conjugates in the extracts of red clover were identified based on the following criteria: (i) measured UV spectra and MS data; (ii) elution order; (iii) behaviour after acid hydrolysis; (iv) literature data.

The characteristic UV absorption spectra of flavonoid compounds exhibit two absorption bands at 240–280 nm and at 300–380 nm [1]. The first absorption band was arisen from the cinnamoyl skeleton and the second band was from the benzoyl skeleton. In addition, the UV spectra of free aglycones, their glucosides and glucoside-malonates are closely similar [19]. Overall 16 isoflavones and their conjugates were tentatively identified by the interpretation of the information obtained from UV spectra. The peaks of the identified compounds are numbered in the chromatogram.

The further confirmative information in terms of accurate masses for both molecular and characteristic fragment ions was obtained from the MS data. As an example, Fig. 3 shows the full-scan ESI-MS spectra of peaks 9, 12 and 16 from the chromatogram in Fig. 2. All spectra contain a predominant ion at *m/z* 283, which corresponds to the deprotonated molecular ion of biochanin A (M_r = 284). Consequently, all three peaks can be attributed to biochanin A and/or their conjugates. The spectrum of peak 9 also contain *m/z* 445, which corresponds to the deprotonated molecular ion of biochanin A glucoside (M_r = 446). Several ions observed at *m/z* higher than 460 most likely can be attributed to an unknown compound which partially co-eluted with biochanin A glucoside. Finally, the MS spectrum of peak 12 displays a minor peak (*m/z* 531) corresponding to the deprotonated molecular ion of biochanin A glucoside malonate (M_r = 532). Thus, on the basis of the obtained data, the peaks can be attributed to biochanin A (peak 16), biochanin A glucoside (peak 9) and biochanin A glucoside malonate (peak 12). A similar analysis of the mass spectra was also performed for other peaks tentatively (from UV spectra) identified as isoflavones or their conjugates. It should be noted that biochanin A and prunetin are constitutional isomers and exhibit identical mass spectra. These compounds were additionally elucidated by a comparison of their retention times with that of the biochanin A standard.

For the particular flavonoid the most polar glucoside form is eluted first following less polar glucoside malonate and the least polar flavonoid aglycone [20]. Consequently, the obtained UPLC elution order provided further valuable information to distinguish between different forms of the particular flavonoid.

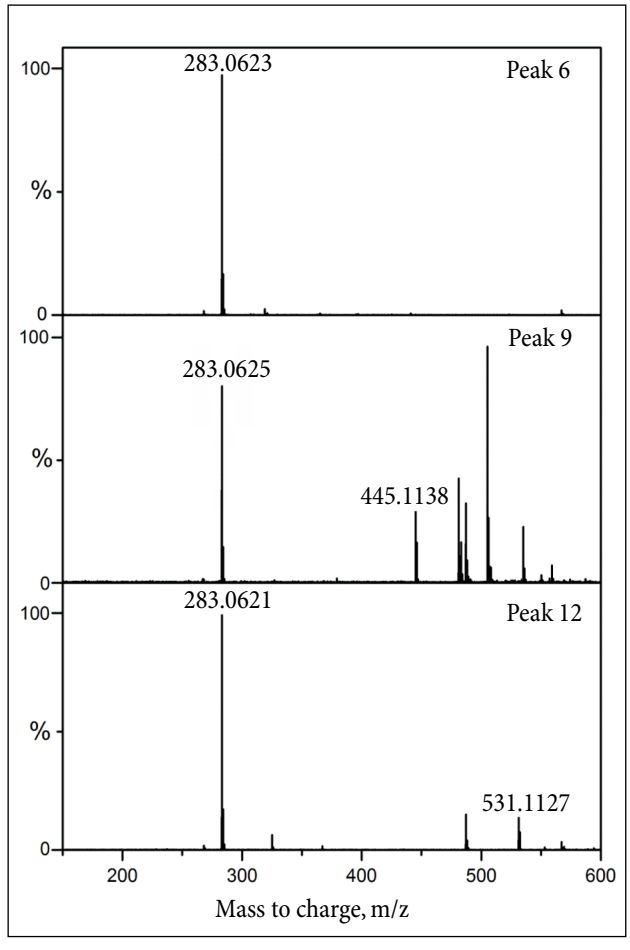

Fig. 3. Full-scan ESI-MS spectra (negative ionization mode) of peaks selected from the chromatogram in Fig. 2

Finally, acid hydrolysis of the extracts was done as an additional confirmation step for the identification of the isoflavones and their conjugated species. During hydrolysis the glucoside and glucoside malonate moieties are removed and conjugated forms of the isoflavone are converted into the corresponding aglycone [20]. Figure 4 shows the UPLC-DAD chromatogram of hydrolyzed red clover extract. As can be observed, several peaks decreased, disappeared or increased in the hydrolyzed sample compared to the unhydrolyzed one. For example, peaks 9 and 12 (see Fig. 2) initially attributed to biochanin A glucoside and biochanin A glucoside malonate species completely disappeared, while peak 16 attributed to biochanin A significantly increased. This served to confirm the identification of biochanin A conjugates. In the same way, the identification of other glucosides and glucoside malonates was also further confirmed by the production of their aglycons in the hydrolyzed extract.

Table 1 summarizes the information obtained from the mass spectra of the isoflavones and their conjugates of red clover. This table includes peak number, retention time, calculated and experimental *m/z* value, error (ppm), mSigma value and possible compound. It should be noted that the used technique does not provide information as regards the position of the glucosyl and malonyl groups. However, previous studies showed that isoflavones in plants were predominantly glucosylated at position 7 and the malonyl group is linked to position 6" of sugar moiety [17, 20]. Only irilone and prunetin, which do not contain an OH group on position 7 are glucosylated at position 4'. The structures of identified compounds are summarized in Table 2.

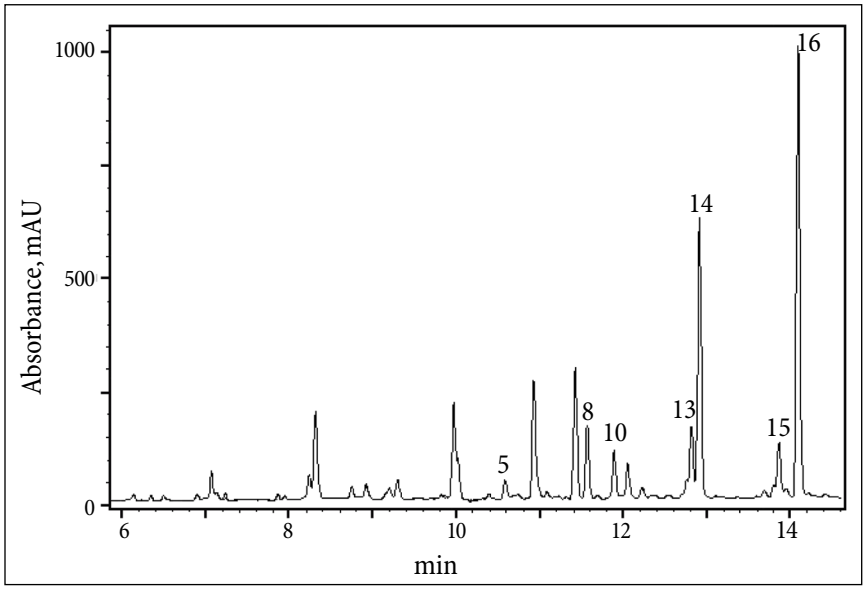

Fig. 4. UPLC-DAD chromatogram of hydrolyzed red clover extract

Table 1. **Data obtained from the mass spectra of the isoflavones and their conjugates of red clover**

Peak number	Retention time, min	Molecular formula	Calculated m/z	Experimental m/z	Error, ppm	mSigma	Possible compound
1	8.96	$C_{21}H_{20}O_{12}$	501.1038	501.1036	0.6	26.6	Daidzein-7-O-glucoside-6″-O-malonate
2	9.73	$C_{24}H_{22}O_{13}$	517.0988	517.0992	–0.9	16.9	Genistein-7-O-glucoside-6″-O-malonate
3	10.17	$C_{25}H_{24}O_{14}$	547.1093	547.1083	1.8	14.6	Pratensein-7-O-glucoside-6″-O-malonate
4	10.41	$C_{22}H_{22}O_{9}$	429.1191	429.1186	1.1	30.5	Formononetin-7-O-glucoside
5	10.62	$C_{15}H_{10}O_{4}$	253.0506	253.0505	0.4	11.8	Daidzein
6	11.16	$C_{25}H_{24}O_{12}$	515.1195	515.1198	–0.7	14.4	Formononetin-7-O-glucoside-6″-O-malonate
7	11.34	$C_{25}H_{22}O_{14}$	545.0937	545.0963	–4.9	24.2	Irilone-4′-O-glucoside-6″-O-malonate
8	11.59	$C_{15}H_{10}O_{9}$	269.0455	269.0463	–3.0	1.1	Genistein
9	11.71	$C_{22}H_{22}O_{10}$	445.1140	445.1138	0.4	17.2	Biochanin A-7-O-glucoside
10	11.93	$C_{16}H_{12}O_{6}$	299.0561	299.0565	–1.4	10.5	Pratensein
11	12.06	$C_{25}H_{24}O_{13}$	531.0612	531.0614	–0.8	9.0	Prunetin-4′-O-glucoside-6″-O-malonate
12	12.11	$C_{25}H_{24}O_{13}$	531.1144	531.1127	3.2	11.4	Biochanin A-7-O-glucoside-6″-O-malonate
13	12.87	$C_{16}H_{10}O_{6}$	297.0405	297.0405	–0.2	2.3	Irilone
14	12.93	$C_{16}H_{12}O_{4}$	267.0663	267.0672	–3.4	5.4	Formononetin
15	13.82	$C_{16}H_{12}O_{5}$	283.0612	283.0614	–0.8	9.0	Prunetin
16	14.14	$C_{16}H_{12}O_{5}$	283.0612	283.0623	–3.8	5.9	Biochanin A

Table 2. **Structures of identified isoflavones and their conjugates**

Compound	R_1	R_2	R_3	R_4	R_5
Biochanin A	-OH	-H	-OH	-OMe	-H
Prunetin	-OH	-H	-OMe	-OH	-H
Formononetin	-H	-H	-OH	-OMe	-H
Irilone	-OH	$-OCH_2O-$		-OH	-H
Biochanin A-7-O-glucoside-6″-O-malonate	-OH	-H	-OGM	-OMe	-H
Prunetin-4′-O-glucoside-6″-O-malonate	-OH	-H	-OMe	-OGM	-H
Pratensein	-OH	-H	-OH	-OMe	-OH
Biochanin A-7-O-glucoside	-OH	-H	-OG	-OMe	-H
Genistein	-OH	-H	-OH	-OH	-H
Irilone-4′-glucoside-6″-O-malonate	-OH	$-OCH_2O-$		-OGM	-H
Formononetin-7-O-glucoside-6″-O-malonate	-H	-H	-OGM	-OMe	-H
Daidzein	-H	-H	-OH	-OH	-H
Formononetin-7-O-glucoside	-H	-H	-OG	-OMe	-H
Pratensein-7-O-glucoside-6″-O-malonate	-OH	-H	-OGM	-OMe	-OH
Genistein-7-O-glucoside-6″-O-malonate	-OH	-H	-OGM	-OH	-H
Daidzein-7-O-glucoside-6″-O-malonate	-H	-H	-OGM	-OH	-H

G is glucoside; GM is glucoside malonate.

CONCLUSIONS

Ultra performance liquid chromatography coupled with DAD and MS detectors is a powerful tool for characterization of main isoflavone species in red clover extracts. The described technique combines superior resolving power of chromatography with UV spectra and high accurate mass measurements. A total of 16 isoflavones as well as their glucoside and glucoside malonates were identified. The position of glycosyl and malonyl groups cannot be elucidated by MS, but complete structures are suggested when supported by literature data.

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IZOFLAVONŲ IR JŲ DARINIŲ IDENTIFIKAVIMAS RAUDONAJAME DOBILE SKYSČIŲ CHROMATOGRAFIJOS SU DAD IR MS DETEKTORIAIS METODU

S a n t r a u k a

Ultraefektyvioji skysčių chromatografija su DAD ir MS detektoriais pritaikyta izoflavonams ir jų dariniams identifikuoti Lietuvoje auginamame raudonajame dobile (*Trifolium pratense* L.). Tiriamieji junginiai buvo ekstrahuojami metanolio / vandens (9:1, *v/v*) tirpalu 60 min. veikiant ultragarsu kambario temperatūroje. Chromatografinis atskyrimas buvo atliekamas Acquity UPLC BEH C18 kolonėlėje naudojant gradientinę eliuciją metanolio / vandens judria faze su 0,25 % (*v/v*) acto rūgšties priedu. Šešiolika izoflavonų aglikonų, jų gliukozidų ir gliukozidų malonatų buvo identifikuota remiantis UV spektrais, MS duomenimis, eliucijos tvarka bei nehidrolizuotų ir rūgštimi hidrolizuotų ekstraktų eliucijos profilių palyginimu.