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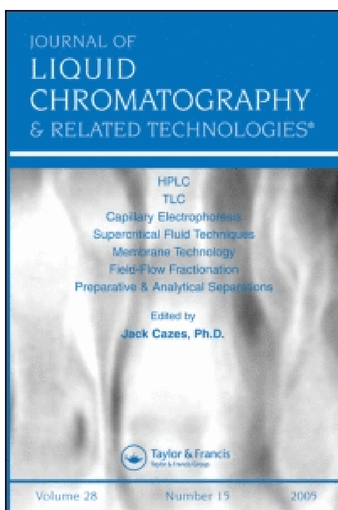

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Determination of Isoflavones in the Aerial Part of Red Clover by HPLC–Diode Array Detection

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Abstract: Red clover (*Trifolium pratense* L.) is a biennial plant which has been used as feeding material for ruminants, but also as a health food for humans due to its estrogenic, antispasmodic, and expectorant properties. Red clover contains a large number of flavones, the four most important being daidzein and genistein and their precursors formononetin and biochanin A, respectively. The purpose of the current project was to quantify these four isoflavones in the aerial part of red clover in samples collected at the flowering, vegetative, and fruiting stages, and accordingly, to determine which of the three growth stages of red clover, contains the highest isoflavone amount. Thus a method based on reversed-phase high performance liquid chromatography (HPLC) using diode array detection has been developed and validated. The linearity, precision, accuracy, and sensitivity of the method allow for the fast and reliable determination of the aforementioned substances from the aerial part of red clover. Analysis of the plant at different growth stages showed that the highest amount of isoflavones was detected during the vegetative stage.

Keywords: Isoflavones, Red clover, HPLC DAD

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INTRODUCTION

Red clover (*Trifolium pratense* L.), Leguminosae family, is a biennial plant grown worldwide. It is an important feeding material for ruminants and a health food for humans due to its estrogenic, antispasmodic, and expectorant properties.[1,2] Flavones are polyphenolic compounds based on the chromane skeleton with a second benzene substitution at the pyran nucleus. They are usually found in plants as glycosides, connected to sugar units such as galactose, rhamnose, glucose, or glucoside malonates. The malonates are a conjugated form of flavones, which is being used by plants to store the less soluble flavone aglycons. Upon microbial infection, the aglycons are liberated from these conjugated forms by hydrolysis.[3,4]

There are six main subgroups of flavones namely the chalcones, flavones, flavonols, flavanones, anthocyanins, and isoflavones. The last subgroup has rather limited taxonomic distribution and it is found mainly within the *Leguminosae* family. Chemically, they are structured by the chromane skeleton with a phenyl substituent at the C_3 position (Figure 1). Biochemicaly they result from flavanones through the phenylpropanoid pathway. The reaction is catalyzed by the isoflavone synthase, a cytochrome P450 dependent monooxygenase found in legumes and a few other species, and has the unique enzymatic function that carries out a 2,3 migration of the B-ring. They are common precursors of phytoalexins (the isoflavone daidzein is the precursor of the major phytoalexins including medicarpin and glyceollins and the isoflavone genistein, which has antifungal activity, is the precursor to the phytoalexin kievitone)[5,6] and phytoanticipins promoting some protection of plants from pathogens. Furthermore, it has been shown that they act as chemoattractants (substances signalling the bacteria to associate with the roots) in the establishment of the symbiosis between the plant and rhizobial bacteria[7] and inducers of nod gene expression.

Isoflavones (Figure 1) are more restricted in nature compared to the flavones and they exert a plethora of biological actions when consumed as part of the human diet, and they also represent the main class of phytoestrogens of current interest in clinical nutrition. Isoflavone phytoestrogens, due

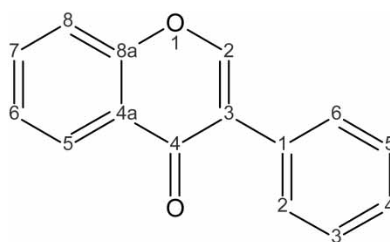

Figure 1. Backbone structure of isoflavones.

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to their structural similarity to the estrogen 17-β-estradiol, have been found to exert potential health benefits in age related and hormone dependent diseases. They exhibit estrogenic[8] and antiestrogenic effects,[9,10] antiatherogenic, antioxidant activity, and anticancer effectiveness against breast,[11–13] colon,[14] skin,[15,16] and prostate tumors.[17–21] A major function of isoflavones is the chemoprevention of osteoporosis as there is clinical evidence that they increase bone density.[22,23] Isoflavones have also been used for the treatment of other postmenopausal disorders like hot flashes where they are used as natural alternatives to hormone replacement therapy (HRT). There are also indications that isoflavones could improve cognitive function and aid towards cancer, menopausal symptoms, cardiovascular disease, and osteoporosis.[24–26]

In red clover, the majority of the estrogenic aglycones are formononetin and biochanin A, with smaller concentrations of daidzein and genistein (Figure 2). Formononetin and biochanin A are precursors of daidzein and genistein, respectively.[24] Several methods have been reported for the determination of the isoflavone content in red clover using high performance liquid chromatography,[1,3,24,27–30] both qualitative,[1,24,29,30] and quantitative.[3,24,27,28] Isoflavones have been determined either in red clover leaves, flowers, stems, or roots, mainly collected at the flowering stage.

The purpose of this study was to quantify the four most important bioactive isoflavones in the aerial part of red clover from samples collected at the flowering, vegetative, and fruiting stages, thus determining which of the three growth stages of red clover contains the highest isoflavone amount. A parallel goal was to establish a faster and more sensitive analytical methodology than the ones already reported.

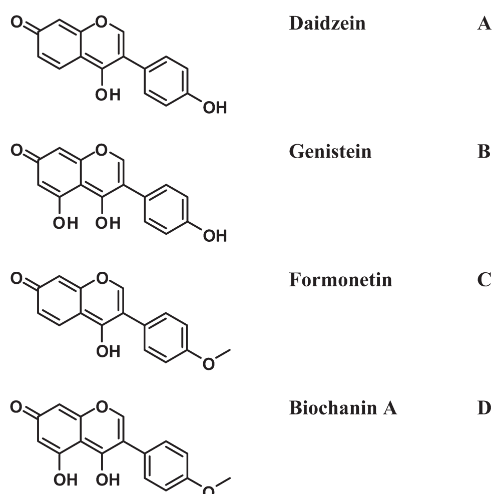

Figure 2. Chemical structures of the four isoflavones under study.

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EXPERIMENTAL

Reagents and Chemicals

Genistein and daidzein were purchased from Alexis Biochemicals (Switzerland), biochanin A from Sigma-Aldrich (MO, USA), and formononetin from Extrasynthése (Genay, France) and were used without further purification. Hydrochloric and phosphoric acids and all organic solvents used throughout the experiments were obtained from Merck (Darmstadt, Germany). Water was purified by reversed osmosis followed by a four column system filtration by means of a Barnstead Nano-Pure Infinity System. The purity of all organic substances was checked by HPLC prior to their use.

Stock Standard Solutions

Stock standard solutions of all analytes were prepared in ethanol at the 1 mg/mL level. Working standard solutions containing daidzein, genistein, formononetin, and biochanin A were prepared by diluting appropriate volumes of the corresponding stock standard solutions in aq. H_3PO_4 (1% v/v) at the 100 μg/mL level.

Sample Collection and Preparation

Samples of the aerial part of red clover (*Salino* variety) were collected from plants grown in the field of the Agricultural University of Athens during the vegetative, flowering, and fruiting stages. The samples were prepared according to the following procedure: 10 mg of air dried pulverized tissue were mixed with 700 μL methanol, 100 μL HPLC-grade water, and 100 μL concentrated HCl (37%), and the mixture was refluxed at 90°C for 1 hour. Consequently, the mixture was left to cool at room temperature. After gravity filtration of the insoluble material through a Whatman Grade 1 filter paper, the extract was diluted with 10 mL of aqueous H_3PO_4 (1:99 H_3PO_4:H_2O v/v), vortexed for 5 min and then an aliquot of 100 μL was used for the HPLC for analysis.

Instrumentation

The HPLC system used for the analysis consisted of a Finnigan Spectra P4000 (Finnigan, Riviera Beach, FL, USA) quaternary pump equipped with 7725i injector (Rheodyne, Rohnert Park, CA, USA) fitted with a 100 μL loop, a Finnigan on-line degasser, and a Finnigan Spectra UV6000LP diode array detector. The whole process was computer controlled by the CHROMQUEST v.2.51 software through a Finnigan SN4000 controller. Data handling and

analysis were carried out by the built in capabilities of the aforementioned software.

Chromatographic separation was performed on a None Porous Silica (NPS) column (53×4.6 mm, I.D. 1.5 μm) (MICRA Bischoff Chromatography), preceded by an on-line filter for the removal of any particulate matter. The column was maintained at 40°C throughout all experiments with the aid of an electronically controlled oven.

HPLC

Gradient elution has been used for the separation of the four substances under analysis using the elution program described in Table 1. Solvent A was HPLC grade water containing 1% (v/v) H_3PO_4 and solvent B was acetonitrile containing 1% (v/v) H_3PO_4. The flow rate was kept at 0.5 mL/min throughout all experiments. The total chromatographic analysis time was 15 minutes. All mobile phases were filtered under vacuum through a 0.2 μm Titan membrane filter (Scientific Resources, USA).

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Detection

The corresponding UV spectra of all four substances were recorded using the diode array detector. The absorbance maxima of the analytes were determined to be 254 nm, therefore, the acquisition of the chromatograms was performed at that wavelength. Identification of the eluting peaks was achieved primarily by comparison of their retention times (t_R) to the corresponding ones obtained by the injection of standard solutions (the time window for peak recognition and assignment has been set to 2% of t_R). The UV spectra of the processed samples obtained at the expected retention times (obtained from the diode array data) were compared to those of the corresponding standard solution and the calculated peak purity was also used as additional evidence towards the identification of the eluting substances.

Table 1. Gradient elution program for reversed-phase high performance liquid chromatographic separation of daidzein, genistein, formononetin and biochanin A

Time (min)	Solvent A[a] (%)	Solvent B[b] (%)	Flow-rate (mL/min)
0	95	5	0.5
12	70	30	0.5
13.2	95	5	0.5
15	95	5	0.5

[a]Solvent A: HPLC - grade water (1% v/v H_3PO_4).

[b]Solvent B: acetonitrile (1% v/v H_3PO_4). Chromatography was performed on a NPS column (53×4.6 mm, particle diameter 1.5 μm).

Validation of the Assay Method

Linearity

For the linearity assay six standard mixtures of the four substances (containing 0.01, 0.05, 0.1, 0.5, 1, and 2 μg/mL of daidzein, genistein, formononetin, and biochanin A) were analyzed. The linearity of the data was evaluated using linear regression analysis.

Precision

The intra-day precision of the overall procedure was determined by analyzing five replicates of the four substance mixture at two concentration levels (0.01 and 0.5 μg/mL) and calculating the corresponding %RSD values. The inter-day precision was determined the same way by analyzing four replicates at the 0.01 μg/mL and 0.5 μg/mL levels in four different laboratory days and calculating the corresponding %RSD values.

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Accuracy

The accuracy of the method was assessed by analyzing a mixture of the four isoflavones at the 0.04 μg/mL and 0.7 μg/mL concentration levels and evaluated as the relative percentage error (%Er).

Recovery

As there are no matrix samples lacking the analyzed isoflavones, the recovery has been calculated using the standard addition methodology as follows: 10 mg of dried pulverized tissue collected at the flowering stage of red clover were mixed with 700 μL methanol, 100 μL HPLC grade water, and 100 μL concentrated HCl (37%). Then the mixture was spiked with either 5 μL or 20 μL of the stock standard solution of isoflavones at the 100 μg/mL concentration level, refluxed at 90°C for 1 hour and then the volume was adjusted to 1 mL with HPLC-grade water. Thus, the final added concentration for each isoflavone corresponds to 0.5 μg/mL and 2 μg/mL (pre-spiked sample, $n = 5$). On the other hand, the same amount of the stock standard solution of isoflavones has been added to the mixture after the reflux (post spiked sample, $n = 5$). The basal levels of isoflavones have been determined according to the procedure described in the sample collection and preparation section (basal concentration). Accordingly, the difference at the concentration before and after the spiking (which is the concentration spiked) has been calculated and compared to the actual concentration added to the sample.

All samples were analyzed using the described methodology. Peak areas were employed for the calculation of concentration. The concentration of the

Table 2. Calibration curves for daidzein, genistein, formononetin and biochanin A

Analyte	Regression equation	Correlation coefficient, r^2
Daidzein	$y = 4693660x + 73891$	0.9992
Genistein	$y = 5352891x + 122343$	0.996
Formononetin	$y = 2147020x + 147307$	0.996
Biochanin A	$y = 5167566x + 176049$	0.996

isoflavones in the matrix has been determined using the equations referred in Table 2 and the recovery R has been calculated as:

$$R = C_{pre-spiked} - C_{basal}/C_{post-spiked} - C_{basal} \quad (1)$$

where $C_{pre\text{-}spiked}$ is the mean value of the analyte concentrations in the pre-spiked samples, C_{basal} is the mean value of the analyte concentrations occurring naturally in the matrix, and $C_{post\text{-}spiked}$ is the mean value of analyte concentrations in the post-spiked samples. The precision of the recovery process is expressed as the %RSD value.

Limits of Detection and Quantitation

The signal-to-noise (S/N) ratio of 3:1 (peak area ratio of the analyte vs. baseline noise) and 10:1 were used for the calculation of the LOD and LOQ, respectively. A 20 sec window adjacent to each peak under consideration was employed for the estimation of the baseline noise in every case.

RESULTS AND DISCUSSION

The chromatographic separation of the four isoflavones is depicted in Figure 3. The chromatogram shows baseline resolution of the compounds with acceptable peak shape. The corresponding t_R's are 5.62 min for Daidzein, 7.16 min for Genistein, 8.78 min for Formononetin, and 11.16 min for Biochanin A. Figure 4 shows the chromatograms obtained from samples of the plant at the flowering, vegetative, and fruiting stages. All substances are baseline separated.

Method Validation

The developed methodology has been validated for its linearity, precision, accuracy, recovery, and sensitivity. Samples have been analyzed immediately

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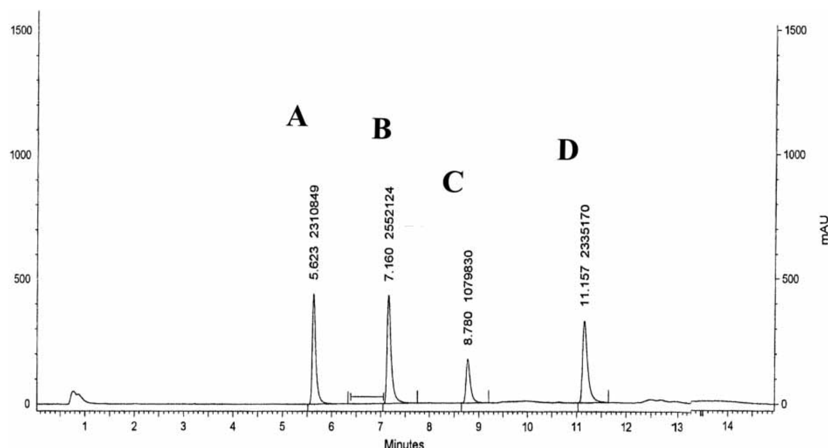

Figure 3. Chromatographic separation of an isoflavone standard mixture at a concentration of 0.5 μg/mL level. A: daidzein r_t, B: genistein r_t, C: formononetin r_t, and D: biochanin A. The retention time and chromatographic peak area are indicated on the top of the peaks.

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after the sample preparation procedure according to the aforementioned sample preparation procedure. All calculations were based on the relevant peak areas of the compounds under study.

Linearity

The linearity of the method was evaluated by linear regression analysis using six concentrations of the four analyte standard mixtures, by the external standard method. Neither weighting nor forcing or including the 0,0 point was used for the construction of the calibration curve. The regression equations calculated for every substance are shown in Table 2, where the respective correlation coefficients r^2 were better than 0.99 in every case. The linearity region was estimated to be in the range of 0.01–2 μg/mL for all substances analyzed. Overall, the results show good linearity for all four analytes.

Precision

The intra-day as well as the inter-day precision of the developed methodology has been determined analyzing five replicates at two concentration levels, namely the LOQ and a middle concentration level (0.01 and 0.5 μg/mL). The intra-day precision, expressed as the %RSD was 5.8% for daidzein, 4.8% for genistein, 4.9% for formononetin, and 10.25% for biochanin A in the 0.01 μg/mL concentration level, and 1.7%, 0.8%, 5.7% and 2.9%,

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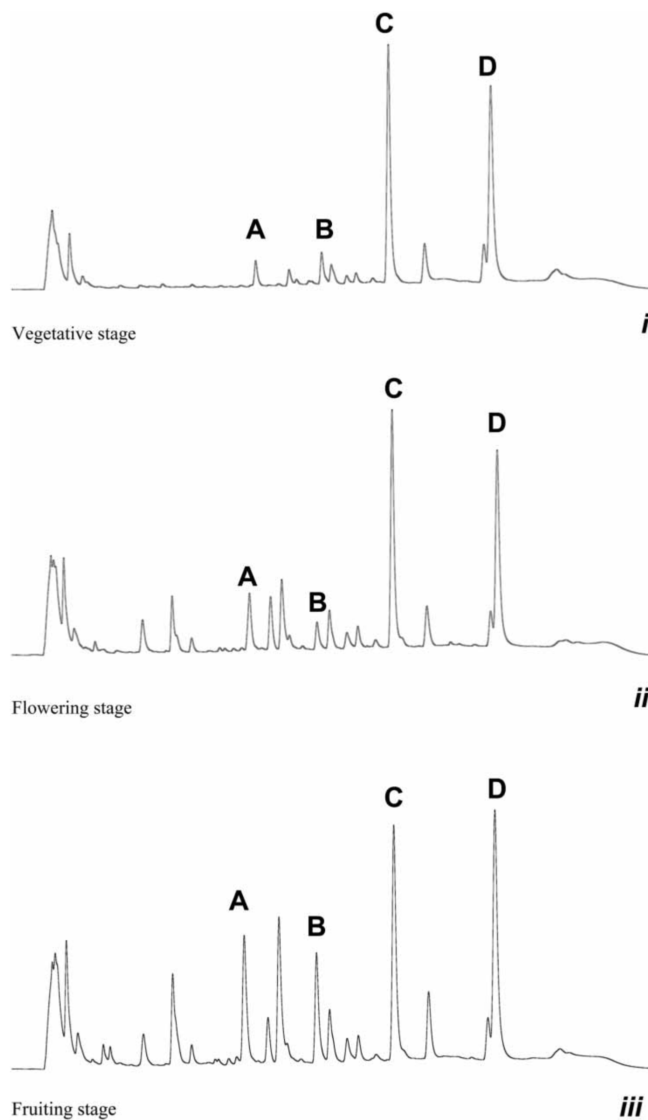

Figure 4. Chromatograms of red clover extracts in three different growth stages: i) vegetative ii) flowering and iii) fruiting stage. A: daidzein, B: genistein, C: formononetin, and D: biochanin A.

respectively, at the 0.5 μg/mL concentration level. The inter-day precision was estimated by assaying samples at the 0.01 μg/mL and the 0.5 μg/mL concentration level at four different laboratory days. The intra day precision was also expressed as the % relative standard deviation (%RSD) and was

found to be 6.4% for daidzein, 7.2% for genistein, 8.8% for formononetin, and 8.7% for biochanin A for the 0.01 μg/mL level; 2.1% for daidzein, 3.1% for genistein, 4.5% for formononetin, and 2.4% for biochanin A for the 0.5 μg/mL level. The aforementioned RSD values indicate that the method is suitable for the analysis of flavones from red clover.

Accuracy

In order to determine the accuracy of the method a mixture of the four substances at the 0.04 and 0.7 μg/mL concentration levels were analyzed. Accuracy was expressed as the relative percentage error ($\%E_r$) defined as:

$$\%E_r = [\text{assayed conc} - \text{nominal conc}]/[\text{nominal conc}]^{*}100$$

The estimated %Er values were and 5.4%, 7.3%, 8.5%, and 7.7% for daidzein, genistein, formononetin, and biochanin A at the 0.04 μg/mL concentration level and 0.7%, 1.4%, 13.2%, and 3.7% f at the 0.07 μg/mL concentration level, thus showing that the method could be considered accurate for bioanalytical purposes.

Recovery

The basal levels of the four isoflavones (C_{basal}) for the flowering stage are shown in Table 3. The flowering stage of the plant has been chosen, because the isoflavone concentration during that period is the lowest compared to the other two periods (vegetative and fruiting), and thus the results obtained will be more credible. The mean recovery values were calculated using the $C_{pre\text{-}spiked}$ and $C_{post\text{-}spiked}$ mean values employing Equation (1) (Recovery section). The mean recoveries were found to be 101 (±4.6) % for daidzein, 87 (±7.4) % for genistein, 72 (±6.5) % for formononetin, and 80 (±10.3) % for biochanin A at the 0.5 μg/mL level and 97 (±3.5) % for daidzein, 91 (±4.4) % for genistein, 76 (±3.5) % for formononetin, and 84 (±6.3) % for biochanin A at the 2 μg/mL level. All calculated values are acceptable for the purpose of this study.

Table 3. Isoflavone content (mg/g dry weight) of red clover aerial part in different growth stages of the plant

Growth stage	Daidzein	Genistein	Formononetin	Biochanin A	Total (mg/g dry)
Vegetative	0.23	0.22	8.24	4.11	12.8
Flowering	0.63	0.11	6.2	2.82	9.76
Fruiting	1.7	1.07	6.2	3.45	12.42

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Sensitivity

The sensitivity of the method as expressed by its limit of detection (LOD) was found to be 0.0033 μg/mL for all substances. The LOQ (the lower concentration of the calibration curve) was 0.01 μg/mL for all substances as well. These values have been calculated using an S/N ratio of 3 and 10 for LOD and LOQ, respectively.

Analysis of Red Clover Samples

The developed methodology has been applied to the determination of the isoflavone content of red clover. The greatest amount of isoflavones was detected in red clover tissues collected at the vegetative stage (Table 3). The aerial part of red clover at the vegetative stage contained 12.8 mg isoflavones/g dry weight, the corresponding one at the fruiting stage contained 12.42 mg isoflavones/g dry weight, whereas the last one at the flowering stage contained 9.76 mg isoflavones/g dry weight. Formononetin and biochanin A were the major components of red clover (formononetin being in higher concentration than biochanin A, in all growth stages), while daidzein and genistein were the minor ones.

CONCLUSIONS

To the best of our knowledge, this is the first attempt for the quantitative determination of red clover isoflavones at the various growth stages of the plant. The highest amount of flavones was detected in red clover collected at the vegetative stage. At this stage the isoflavone content was 12.8 mg/g dry weight (8.4 mg/g formononetin, 4.11 mg/g biochanin A, 0.23 mg/g daidzein, and 0.22 mg/g genistein). According to the data of a previously reported study, the total isoflavone content (5.14 mg/g), as well as the content of each isoflavone (2.89 mg/g formononetin, 2.04 mg/g biochanin A, 0.11 mg/g daidzein, and 0.10 mg/g genistein), was significantly lower.[3] At the flowering stage, the isoflavone content was 9.76 mg/g dry weight (6.2 mg/g formononetin, 2.82 mg/g biochanin A, 0.63 mg/g daidzein, and 0.11 mg/g genistein). These concentrations are in agreement with those calculated during a previous study (0.5–2.07 mg/g formononetin, 0.99–2.28 mg/g biochanin A, 0.71–1.93 mg/g daidzein, and 0.9–7.99 mg/g genistein), except for formononetin which has been found at a much lower concentration.[24] At the fruiting stage the isoflavone content was 12.42 mg/g dry weight (6.2 mg/g formononetin, 3.45 mg/g biochanin A, 1.7 mg/g daidzein, and 1.07 mg/g genistein). Thus, formononetin and biochanin A were determined to be the major components of red clover (formononetin being in higher concentration than biochanin A in all growth stages), while

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daidzein and genistein were the minor ones. It should be noticed, that the highest concentrations of formononetin and biochanin A were found at the vegetative stage of red clover, while the highest concentrations of daidzein and genistein at the fruiting stage.

The developed analytical methodology has been shown to be accurate, sensitive, precise, and reliable. The time of each analysis was much shorter (only 15 minutes) compared to the time analysis of previous studies (61 min,[27] 40 min[24] and 42 min[3]), mainly due to the use of a shorter column (53×4.6 mm, I.D. 1.5 μm) with a small particle size and greater separation capacity. The selected column proved to have greater separation capacity than the conventional columns, which in combination with the method's elution program yielded well resolved peaks in the resulting chromatograms.

The LOQ of the validated analytical method is 0.01 μg/mL, thus demonstrating higher sensitivity compared to the corresponding values of previous methods (0.026–0.032 μg/mL,[24] 20–90 μg/mL[3]). This study has established a framework for evaluating the isoflavone content not only in different red clover parts but also in samples of different red clover growth stages. Similarly, this methodology could be useful as a quality control method for screening various growth stages of other *Trifolium* plants for their flavone content.

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