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Quantification of isoflavones in red clover by high-performance liquid chromatography

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Abstract

An RP–HPLC method for the determination of daidzein, genistein, formononetin and biochanin A in red clover (*Trifolium pratense* L.) was developed and validated. The compounds are quantified after hydrolytic extraction using an internal standard. On a base-deactivated C_{18} column good separation of the analytes, also from accompanying substances, and excellent peak shape are achieved by gradient elution with aqueous sulfuric acid and acetonitrile. The method was applied to the analysis of different red clover cultivars.

Keywords: Red clover; Isoflavones

1. Introduction

Epidemiologic observations as well as animal studies and some clinical trials in humans suggest that dietary phytoestrogens have a high potential in the prevention of 'Western diseases' like atherosclerosis, osteoporosis, cardiovascular diseases, several kinds of cancer and postmenopausal complaints [1–5]. One important group of phytoestrogens are isoflavones like daidzein and genistein, which occur in soy and soy products, to which most of the epidemiologic studies on beneficial health effects of phytoestrogens refer. Due to these effects the demand for food supplements rich in isoflavones is strongly increasing. Red clover, a fodder plant, which is grown widely in Europe, contains closely related isoflavone glycosides, mainly of the genins formononetin and biochanin A, besides smaller amounts of daidzein and genistein glycosides [6]. In humans the glycosides are hydrolyzed and formononetin and biochanin A are metabolized to daidzein and genistein [2]. Thus the plant is a cheap and easily available source for the production of isoflavone-rich food supplements for women suffering under menopausal complaints.

For the selection of the optimal plant material in the development of such a food supplement an analytical method for the quantification of the isoflavones was necessary. For the determination of isoflavones, mainly HPLC is used. Numerous methods have been published for the quantification of soya isoflavones, focusing on daidzein, genistein, eventually biochanin A and their glycosides [7–12]. Due to the differences in the accompanying substances these systems were not suitable for red clover. Some HPLC methods developed for the determination of isoflavones in clover have disadvantages like the determination of only three of the analytes [13] or are quite time consuming in sample

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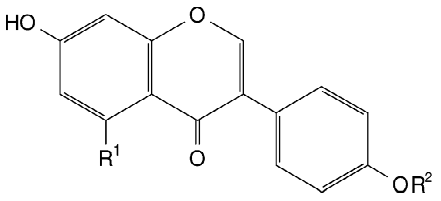

Daidzein	R^1 = H	R^2 = H
Genistein	R^1 = OH	R^2 = H
Formononetin	R^1 = H	R^2 = CH_3
Biochanin A	R^1 = OH	R^2 = CH_3

Fig. 1. Structures of the analytes.

preparation [14–18]. The most important requirement for the method needed was a simple, fast and cheap sample preparation, which had priority upon a short time of analysis. To facilitate the quantification the hydrolysis of the isoflavone glycosides during extraction and determination of the four genins, daidzein, genistein, formononetin and biochanin A (see Fig. 1), with the use of an internal standard was demanded. As the comparison of buffered and unbuffered mobile phases in HPLC of red clover isoflavones had shown better results without buffer [17], in the proposed method a buffer system in the eluent was avoided. Based on these parameters a respective method was developed and validated.

2. Experimental

2.1. General

2.1.1. Chemicals

Daidzein, genistein, and biochanin A were purchased from Sigma Chemical Co. (St. Louis, USA), 6-methoxyflavanone from Aldrich Chemical Co. (Milwaukee, USA), formononetin from Carl Roth AG (Karlsruhe, Germany). Acetonitrile (HPLC grade) and trifluoroacetic acid (analytical reagent grade) were obtained from Merck (Darmstadt, Germany). All other chemicals were analytical reagent grade.

2.1.2. Equipment

HPLC was performed on a Merck Hitachi system consisting of a LaChrom pump L-7100, a programmable autosampler L-7250, an interface D-7000 and a LaChrom diode array detector L-7450 (Merck, Vienna, Austria).

2.2. Column liquid chromatography

For separation of the isoflavones a 250×4 mm I.D. Hypersil BDS-C_{18} column with 5-μm particles (Shandon, Runcorn, UK) was operated at room temperature.

The eluent consisted of water, adjusted with sulfuric acid to pH 2.7 (A) and acetonitrile (B). The gradient profile was: 0–35 min from 20 to 37% B, 35–45 min from 37 to 100% B, 45–50 min 100% B, 50–51 min 100 to 20% B, 51–61 min 20% B. The flow-rate was 1 ml/min. The wavelength of detection was 254 nm.

2.3. Standard calibration

From stock solutions of 1.5 mg/ml daidzein, 1.5 mg/ml genistein, 1.8 mg/ml formononetin, 0.35 mg/ml biochanin A and 3.3 mg/ml 6-methoxyflavanone (internal standard) standard mixtures were prepared. For all solutions dimethylsulfoxide was used as solvent. The resulting mixtures were stored at 4 °C. Calibration graphs were obtained using five mixtures with all standards at different concentrations. Ten-μl volumes of these solutions were analysed in duplicate.

2.4. Samples and sample preparation

Different samples of red clover and red clover extracts were obtained from Melbrosin International (Vienna, Austria).

Two-hundred mg of pulverized drug were extracted with 30 ml 80% methanol (acidified to pH 3 with trifluoroacetic acid) for 15 min under reflux at 85 °C. After filtration and washing of the residue with 20 ml 80% methanol the extract was evaporated under reduced pressure. The extract was dissolved in 2.00 ml dimethylsulfoxide; to 500 μl of the solution 40 μl of standard solution (15.0 mg 6-methoxyflavanone/ml) were added and 10 μl of the mixture were analysed.

Ten mg of red clover extract were dissolved in 1.00 ml dimethylsulfoxide–water (3+1). To 500 μl

of the solution 40 μl of standard solution (15.0 mg 6-methoxyflavanone/ml) were added and 10 μl of the mixture were analysed.

3. Results and discussion

The requirements for the new method for the investigation of isoflavones in red clover were a simple sample preparation including the entire hydrolysis of the isoflavone glycosides and the quantification of the resulting aglyca biochanin A, formononetin, genistein and daidzein by the use of an internal standard.

3.1. Sample preparation

Usually hydrochloric acid is added to achieve complete hydrolysis of the isoflavone glycosides in red clover during the extraction with ethanol or methanol [14,17,18]. In the reported method the drug was extracted with 80% methanol acidified with trifluoroacetic acid. As trifluoroacetic acid is completely removed during evaporation, no additional neutralization step was necessary. The extraction procedure was tested at different pH values (pH=1, 2, 3 and 4, respectively). In all analyses other than the one with solvent of pH=1 the same amount of isoflavones was extracted from the drug, whereas at the lowest pH minimal degradation of the compounds was observed. Additionally, under very acidic conditions (pH 1 and 2) higher amounts of accompanying substances were hydrolyzed and extracted, leading to much more impure extracts. Thus, the extraction was performed with solvent of pH=3. Investigations to optimize the extraction time showed that after 15 min reflux at 85 °C exhaustive hydrolysis and extraction was achieved (see Table 1).

The quantitative extraction of biochanin A and formononetin was proved by a recovery test for each substance. A red clover sample of known content was spiked with a defined amount of the two isoflavones, respectively, and sample preparation and HPLC were performed as described (see Experimental). The determination was repeated twice. Mean recoveries were 99.2% for biochanin A and 98.2% for formononetin. As daidzein and genistein occur only in very small amounts in red clover, no recovery assays were performed for these compounds.

Table 1
Isoflavone content of a red clover sample in dependence of extraction time

Compound / Extraction time	Amount (mg/g)		
	15 min[a]	30 min[b]	60 min[b]
Daidzein	0.11	0.10	0.10
Genistein	0.10	0.07	0.08
Biochanin A	2.04	1.89	2.03
Formononetin	2.89	2.65	2.85
Total isoflavone content	5.14	4.71	5.06

[a] Data are means from 13 analyses.
[b] Data are means from two analyses.

The advantages of the described extraction method are the simple handling, low price and low time consumption. In repeated (n=~400) HPLC analyses of these extracts no problems occurred due to accompanying substances.

3.2. Chromatographic conditions

In HPLC-analysis of isoflavones in clover usually RP C_{18} phases are used [16–18]. Due to excellent experiences with base deactivated C_{18} phases for the separation of phenolic compounds [19–21] a Hypersil BDS RP-C_{18} (250×4 mm, 5 μm) column was applied to the system. To avoid buffer systems [17], acetonitrile and aqueous sulfuric acid were used as mobile phase (see Experimental).

As one main requirement of the new method was the simple and cheap sample preparation, for a sufficient separation of the four analytes from accompanying substances gradient elution had to be applied. Figs. 2 and 3 show the typical separation of a standard mixture and an extract from clover obtained under the optimized chromatographic conditions.

Intra-day as well as inter-day reproducibility of retention times was excellent (see Tables 2 and 3). The hold-up time for calculation of the capacity factors was estimated using the clear solvent front peak.

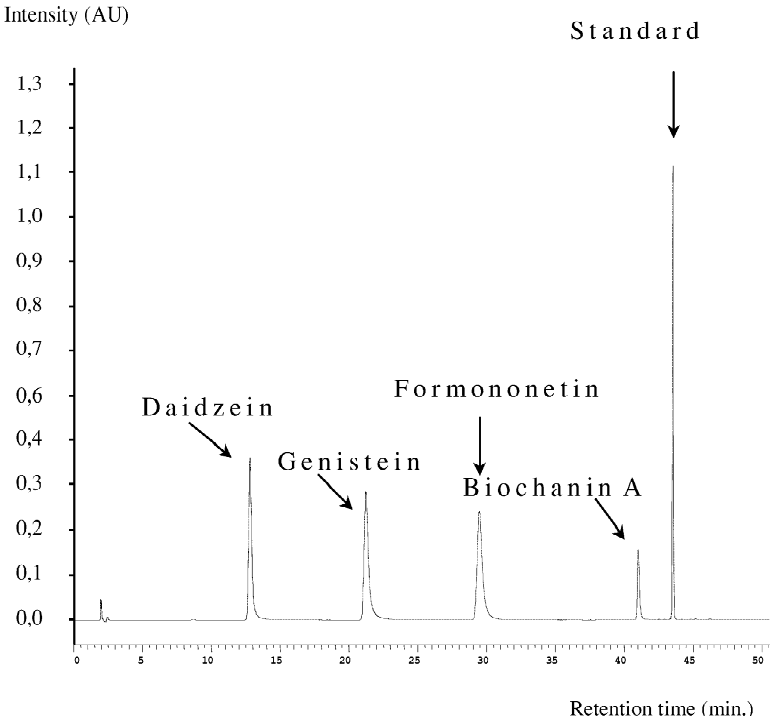

Fig. 2. HPLC of a standard mixture containing 15 μg/ml genistein and daidzein each, 18 μg/ml formononetin, 3.5 μg/ml biochanin A and 33 μg/ml 6-methoxyflavanone as internal standard.

3.3. Quantitative analysis

Several substances e.g. acacetin, chrysin, naringenin, 6-hydroxyflavone, 6-hydroxyflavanone were tested for their applicability as internal standard and 6-methoxyflavanone proved to be the optimal one. This substance elongates analysis time only marginally, is commercially available and shows a UV maximum at 254 nm, the wavelength used for detection.

The system was calibrated for the four isoflavones. Over the selected range, peak areas linearly depended on concentrations for all compounds with correlation coefficients of >0.9988 (see Table 4). The calibration ranges adequately covered the variations in amounts of isoflavones in the samples. The reproducibility of the method was determined by 13-fold determination of a red clover sample (see Table 5). Additionally the total isoflavone content of a commercial red clover extract (declaration 8% total isoflavones) was determined periodically for inter-day control of the HPLC system, 29-fold assay of this extract over a period of 1 year resulted in a mean value of $8.083 \pm 0.175\%$ with a relative standard deviation of 2.17%. These standard deviations prove the excellent accuracy and reproducibility of the method. The limits of detection were calculated experimentally at signal-to-noise ratios of 3 to 1 (see Table 4).

3.4. Analysis of red clover samples

With the new method different red clover cultivars were analysed for the determination of the best material for the production of an isoflavone-rich extract. In all samples formononetin and biochanin A were the main compounds, while only small amounts of daidzein and genistein were detected. The content of biochanin A and formononetin varied from 0.025 to 0.3%, the daidzein and genistein concentrations lay at about one tenth of these values, depending on the cultivar and origin.

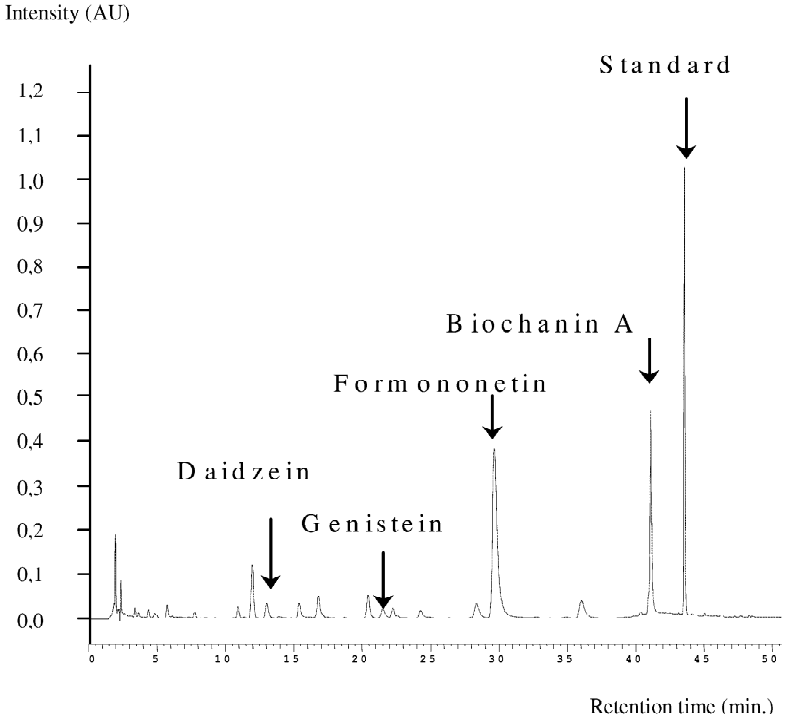

Fig. 3. HPLC of a sample of dried red clover (extraction, see Experimental) internal standard 6-methoxyflavanone.

Table 2
Inter-day reproducibility of retention times

Compound	Retention time (min)	RSD (%)
Daidzein	16.1±0.31	1.91
Genistein	25.4±0.47	1.84
Biochanin A	32.3±0.39	1.22
Formononetin	41.7±0.12	0.29
6-Methoxyflavanone (I.S.)	43.6±0.07	0.16

Data are means from 11 analyses within 2 weeks.

4. Conclusions

The method described enables the quantification of daidzein, genistein, formononetin and biochanin A in red clover after hydrolysis of the respective glycosides. The advantages lie in the simplicity of sample preparation and the low costs of reagents used. The proposed HPLC conditions ensure sufficient resolution and the use of an internal standard guarantees the precise quantification of the compounds. Results

Table 3
HPLC data for the isoflavones and internal standard based on intra-day retention times

Compound	Retention time (min)	RSD (%)	Capacity factor k'	Separation factor α
Daidzein	16.1±0.06	0.39	7.47	–
Genistein	25.5±0.09	0.35	12.42	1.66
Biochanin A	32.4±0.09	0.30	16.05	1.29
Formononetin	41.7±0.02	0.05	20.95	1.31
6-Methoxyflavanone (I.S.)	43.6±0.02	0.04	21.95	1.05

Data are means from 10 analyses

Table 4
Characteristics of the analytical method derived from the standard calibration set

Compound	Detection limit ng	Quantification limit[a] ng	Linear range mg/l	Correlation coefficient
Daidzein	1.6	5.0	1.0–260.0	0.9988
Genistein	3.1	8.5	1.5–260.0	1.0000
Biochanin A	0.6	2.0	0.5–60.0	0.9999
Formononetin	3.7	10.0	2.0–300.0	0.9998

[a] The limit of quantification was defined as the amount of analyte showing a signal-to-noise ratio of 10 to 1.

Table 5
Reproducibility of the method

Compound	Amount (mg/g)	RSD (%)
Daidzein	0.11±0.0028	2.54
Genistein	0.10±0.0053	5.06
Biochanin A	2.04±0.0554	2.72
Formononetin	2.89±0.0872	3.02
Total isoflavone content	5.09±0.1173	2.30

Data are means from 13 extractions of a sample of red clover.

from statistical analysis of the experimental results are indicative of satisfactory precision and reproducibility.

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