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# Ultrahigh-pressure liquid chromatography of isoflavones and phenolic acids on different stationary phases

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ABSTRACT

Complete separation of aglycones and glucosides of selected isoflavones (genistin, genistein, daidzin, daidzein, glycitin, glycitein, ononin, sissotrin, formononetin, and biochanin A) was possible in 1.5 min using an ultrahigh-pressure liquid chromatography (U-HPLC) on a different particular chemically modified stationary phases with a particle size under 2 μm. In addition, selected separation conditions for simultaneous determination of isoflavones together with a group of phenolic acids (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, and sinapic acid) allowed separation of all 19 compounds in 1.9 min. Separations were conducted on a non-polar reversed phase ($C_{18}$) and also on more polar phases with cyanopropyl or phenyl groups using a gradient elution with a mobile phase consisting of 0.3% aqueous acetic acid and methanol. Chromatographic peaks were characterised using parameters such as resolution, symmetry, selectivity, etc. Individual substances were identified and quantified using UV–vis diode array detector at wavelength 270 nm. Limits of detection (3 S/N) were in the range 200–400 pg $ml^{-1}$. Proposed U-HPLC technique was used for separation of isoflavones and phenolic acids in samples of plant materials (*Trifolium pratense*, *Glycine max*, *Pisum sativum* and *Ononis spinosa*) after acid hydrolysis of the samples and modified Soxhlet extraction.



## 1. Introduction

Due to the importance of phenolic compounds in biomedicine, new isolation, purification and separation techniques are developed in order to increase the speed of isolation and also simplify identification of the compounds in plant materials, food supplements and pharmacological preparations (see reviews [1–4]). Recently a paper dealing with chemistry, physiological effects and analysis of phenolic acids appeared [5] and the recent trends in isolation and identification of isoflavones (mainly in plant materials) have been reviewed [6].

At present liquid chromatography on a reversed phase (RP) is most widely used for the separation of the substances. An octadecyl ($C_{18}$) chemically bound on a silica gel surface belongs to the most often used stationary phase. Very good results (separation of 10 isoflavones in 4 min) were obtained in the case of HPLC with a column filled with a RP sorbent having particle size below 3 μm and using optimised linear gradient elution with aqueous 0.3% acetic acid and acetonitrile and subsequent electrospray MS detection [7]. Separation of a more complicated mixture of isoflavones was impossible in less than 5 min using classical HPLC on the other hand.

In addition to the classical HPLC, new technologies (generally known as ultrahigh-pressure liquid chromatography, U-HPLC) performing separation at elevated pressures (usually over 6000 psi) and/or column temperature and application of particular sorbents with a particle size under 2 μm [8] seem to be very effective. Especially the particle size (but also its geometry) of the sorbents plays the most important role in separation efficiency (see van Deemter's equation and recent paper [9]) and number of theoretical plates can be as high as several 10,000 [10].

Recently we presented a paper describing separation of 10 selected isoflavones with retention times under 60 s and peak half widths around 1 s [10] and their determination in real samples of soy food and plant materials in less than 2 min. Farré et al. [11] separated isoflavones at seriously reduced retention times (under 3.5 min). The very serious advantage of the application of the above-mentioned technologies lies in the very high sample throughput. For example, U-HPLC technique was applied for determination of different groups of phenols (including isoflavones) in aerial parts of *Trifolium* sp. [12] in less than 8.5 min. Comparison of classical HPLC and U-HPLC using electrospray mass spectrometric detection for separation of isoflavones and selected plant metabolites presented serious reduction of retention times and half width of peaks in the

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**Table 1**
Summary of selected isoflavones or other flavonoids U-HPLC separation in different samples

| Compounds | Column | Mobile phase solvents | Detection | Application | References |
|---|---|---|---|---|---|
| Soy isoflavones, tamoxifen metabolites | $C_{18}$/Acquity Waters (50 mm × 1 mm, 1.7 μm) | Acetonitrile/formic acid | MS/MS | UPLC/HPLC comparative study | [13] |
| Isoflavones, phenolic acids and clovamides | $C_{18}$/BEH Waters (50 mm × 2.1 mm, 1.7 μm) | Acetonitrile/acetic acid | UV–vis DAD | Identification and quantification of phenolics in *Trifolium* sp. | [12] |
| Flavonoid glucosides | $C_{18}$/BEH Waters (50 mm × 2.1 mm, 1.7 μm) | Acetonitrile/acetic acid | UV–vis DAD, MS/MS | *Aesculus hippocastanum* seeds analysis | [44] |
| Estrogens, daidzein, genistein, biochanin A | $C_{18}$/Acquity Waters (50 mm × 2.1 mm, 1.7 μm) | Acetonitrile/water | Q-TOF-MS, MS/MS | Wastewater sample monitoring | [11] |
| Isoflavoid aglycones and glucosides | $C_{18}$/Zorbax SB (30 mm × 2.1 mm, 1.8 μm) | Methanol/acetic acid | UV–vis DAD, ESI-MS | *Trifolium pratense*, *Ononis spinosa*, *Iresine herbstii*, soy foods analysis | [10] |
| Flavonoids (orientin, vitextin, etc.) | $C_{18}$/BEH Waters (100 mm × 2.1 mm, 1.7 μm) | Acetonitrile/acetic acid | ESI-MS/MS | *Trollius ledibouri* constituents identification | [45] |

ESI, electrospray ionisation; MS/MS, tandem mass spectrometry; DAD, diode array detection; Q-TOF, quadrupole-time of flight.

case of U-HPLC [13]. These and other applications of U-HPLC for isoflavones separation are described in Table 1.

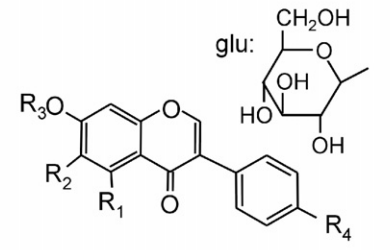
Besides using packed columns with particular sorbents, aglycones and glucosides of isoflavones were also separated using monolithic columns that are characterised by more compact filling. Monolithic $C_{18}$ stationary phase was used for separation of daidzin, genistin, glycitein and their glucosides in soy food samples. Separation of 12 isoflavones after their previous extraction was done in approximately 10 min with very good resolution of chromatographic peaks [14]. Application of a monolithic reversed phase for separation of 11 flavonoid aglycones with total separation times shorter than 15 min [15] belongs to the most recent results.

The aims of the current paper are (i) to find the best conditions for determination of isoflavones (daidzein, genistein and glycitein, their glucosides and other derivatives; see Fig. 1) using octadecyl-, phenyl- and cyanopropyl-modified stationary phases, (ii) to develop an efficient chromatographic procedure for simultaneous separation of selected phenolic acids and isoflavones, and (iii) to apply the method for identification of the substances in acid hydrolysates of plant materials after their previous isolation with modified Soxhlet extraction.

## 2. Materials and methods

### 2.1. Chemicals

HPLC-grade acetic acid and methanol were from Merck (Darmstadt, Germany). Isoflavones, phenolic acids and all other reagents of ACS purity were purchased from Sigma Aldrich (St. Louis, MO, USA). The stock standard solutions of isoflavones and phenolic acids at 10 mg $ml^{-1}$ were prepared in aqueous methanol (1:1, v/v) and stored in dark at 4 °C. The working standard solutions were prepared daily by dilution of the stock solutions with the aqueous methanol (1:1, v/v). All solutions were filtered through a 0.45-μm




| Isoflavones | | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|
| daidzin | daidzein-7-O-β-D-glucopyranoside | H | H | glu | OH |
| glycitin | glycitein-7-O-β-D-glucopyranoside | H | $OCH_3$ | glu | OH |
| genistin | genistein-7-O-β-D-glucopyranoside | OH | H | glu | OH |
| daidzein | 4,7-dihydroxyisoflavone | H | H | H | OH |
| ononin | formononetin-7-O-β-D-glucopyranoside | H | H | glu | $OCH_3$ |
| glycitein | 4,7-dihydroxy-6-methoxyisoflavone | H | $OCH_3$ | H | OH |
| genistein | 4,5,7-trihydroxyisoflavone | OH | H | H | OH |
| sissotrin | biochanin A-7-O-β-D-glucopyranoside | OH | H | glu | $OCH_3$ |
| formononetin | 7-hydroxy-4'-methoxyisoflavone | H | H | H | $OCH_3$ |
| biochanin A | 5,7-dihydroxy-4'-methoxyisoflavone | OH | H | H | $OCH_3$ |

glu: glucopyranosyl

**Fig. 1.** Chemical structure of analysed isoflavones.