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Research Article

# APPLICATION OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY FOR QUALITATIVE ANALYSIS OF ISOFLAVONES IN MEDICINAL PLANTS

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**ABSTRACT**

The retention behavior of commonly occurring compounds with estrogenic activity: daidzein, formononetin, genistein, prunetin, biochanin A and coumestrol on silica and silica modified with cyjanopropyl, aminopropyl, diol and octadecyl groups was investigated. Mixtures of methanol or acetonitrile with water and eluents such as chloroform, heptane and toluene with more polar modifiers: ethyl acetate, acetone, diisopropyl ether in various concentrations were used as mobile phases. The best result of separation was obtained on RP-18s plates with use diisopropyl ether/toluene/formic acid (40/60/0.5 v/v/v) as eluent. All investigated compounds were well separated which enabled their densitometric analysis. The proposed chromatographic system was used to qualitative analysis of investigated compounds in plant material.

**Keywords:** Isoflavones, coumestrol, phytoestrogens, thin layer chromatography, modified silica.

## INTRODUCTION

Isoflavones are derivatives of 3-phenyl-chromen-4-one. They are found in many plants and plant-derived foods in both as aglycon forms and as β-glucosides, acetyl- or malonyl-esters[1]. Red clover (*Trifolium pratense* L.), kudzu (*Pueraria lobata*, Willd.), soy (*Glycine max* L.) and soy products such as soymilk, tofu or miso are especially rich sources of these polyphenols[2]. Recently, these group of natural compounds have gained widespread attention as potential therapeutic agents because of their antioxidant, anibiotic, anti-inflammatory and anti-allergic properties[3]. They protect against some chronic diseases associated with aging, such as osteoporosis, cognitive dysfunction, cardiovascular diseases[1,4,5] and reduce the risk a certain types of cancer [6]. They also possess estrogenic activity associated with their structural similarities to β-estradiols and they are often called “phytoestrogens”. Dietary supplements and herbal preparations containing isoflavones are getting more popular as an alternative treatment for their low toxicity and therapeutic effects.

The most employed method for identify and quantify of isoflavones is high-performance liquid chromatography (HPLC)[7-10], however its main disadvantages are long analysis times, sophisticated instrumentation and high amounts of solvents during the whole validation process. On the other hand, HPTLC is useful and inexpensive tool for the phytochemical assessment of plant extracts and herbal drug formulations[11,12]. It is willingly used especially as a screening method due to high sample-throughput and simple samples preparation without time-consuming process of purification.

There are some publications on HPTLC/TLC analysis of isoflavones on silica[13,14], however according to our knowledge there are no data

about investigation of isoflavones retention on modified adsorbents.
The aim of our work was investigation of diol, aminopropyl, cyjanopropyl, and octadecyl silica for separation the most common phytoestrogens: isoflavone aglycones and coumestrol. The similarities of their chemical structures (Fig.1) can cause difficulty in their separation on silica. Our researches could be useful for screening analysis of these compounds both in plant material and herbal preparations.

## MATERIALS AND METHODS

### Materials and chemicals

Isoflavones standards: daizein, formononetin, genistein, prunetin, biochanin A and coumestrol were supplied by Sigma-Aldrich (Germany).
All solvents: acetonitrile, methanol, chloroform, heptane, ethyl acetate and diizopropyl ether were pro analysis grade from Polish Reagents (POCh, Gliwice, Poland). HPTLC plates were from Merck (Darmstadt, Germany). Red clover (*Trifolium pratense* L), *Genista tinctoria* L., soy (*Glycine max* L.) were purchased from local market.

### Standards and samples preparation

Standard solutions were prepared by dissolving 10 mg standards in 10 mL of methanol.
1.0 g of each plant material was defeated with use of chloroform and next, extracted twice with methanol (2x50 mL) within 30 min. at $40^0$ C in an ultrasonic bath. The obtained extracts were combined and concentrated to 25 mL.
Hydrolysis condition: 2.5 mL of 1.0 mol/L hydrochloric acid was added to 10 mL of each extract and made up of methanol to 25 mL in volumetric flask. Hydrolysis was conducted during 2 hours at $37^0$ C. All samples were neutralized and filtered before use.

### Thin layer chromatography (TLC)

Chromatography was performed on 10 cm x 10 cm $F_{254}$ HPTLC Si 60, diol, CN, $NH_2$ and RP-18 plates. Standards and samples were spotted as 5 mm bands using an automatic applicator Desaga AS 30 (Heidelberg, Germany).
The plates were developed to a distance of 85 mm in chromatographic horizontal Teflon DS chamber (Chromdes Lublin Poland), previously saturated with vapors of the mobile phase. After drying in the stream of warm air, the plates were observed at 254 and 366 nm under UV lamp.

## RESULTS AND DISCUSSION

Chromatographic systems used for TLC analysis of isoflavones are mainly based on silica gel as a stationary phase and mixtures of chloroform or dichloromethane with methanol or ethyl acetate in different proportions as eluents. Initially, in our investigations, mobile phases compositions according to literature [13-15] were tested to separation six commonly occurring compounds with estrogenic activity: five isoflavones and coumestrol.
However, the obtained results were unsatisfactory, in proposed chromatographic systems pairs: prunetin - biochanin A and coumestrol - genistein were poor separated. The $hR_F$ values for investigated compounds are presented in Table 1. Further, mobile phases composed with heptane, toluene, dichloromethane or chloroform with increasing amounts of more polar modifiers: methanol, acetone, ethyl acetate were tested on silica and DIOL-silica. 0.01% of formic acid was added to all eluents in order to improve the shape of chromatographic bands. The best but not satisfactory results were obtained for diisopropyl ether/toluene and ethyl acetate/chloroform on silica HPTLC plates (Fig. 2A, 2B). It could be notice that compounds were divided into three groups. Daizein was well separated from the other components and had the lowest $hR_F$ values, prunetin and biochanin A were the strongest eluted, while formononetin, cumestrol and genistein had the mild retention and were poor separated from each other.
Aminopropyl- and cyjanopropyl propyl silica are less polar than silica and these adsorbents can be used in normal (NP) and reversed phase (RP) system. In NP system, the composition of mobile phases for CN plates were the same as for silica. However, investigated compounds were strongly retained on aminopropyl adsorbent thus more polar solvents were used for elution, such as: chloroform with methanol, acetone and ethyl acetate. In RP system, methanol or acetonitrile with increasing amount of water was tested for both adsorbents. The examples of obtained $hR_F$ values on $NH_2$ and CN plates are presented on Fig. 3. As it can be seen the results in term of selectivity were much worse than on silica.
There are two types of RP18 adsorbent employed in planar chromatography. Their various selectivity is caused by difference in amount of bonded octadecyl groups: RP18s (2.5µmol/$m^2$) and RP18w (0.5µmol/$m^2$). The second type poses relatively high amount of free silanol groups and eluents containing water can be used as a mobile phases.

Although, octadecyl silica is adsorbent typically employed in RP HPLC of isoflavones, its utility in thin layer chromatography occurred poor (Fig. 4).

The best selectivity was obtained on RP-18s plates with use weak polar eluent: diisopropyl ether/toluene/formic acid 40/60/0.5 v/v/v. All investigated compounds were well separated which enabled their densitometric analysis (Fig. 5).

The proposed chromatographic system was successfully used to quality control of phytoestrogens in red clover (*Trifolium pratense* L), *Genista tinctoria* L. and soy (Fig. 6).

## CONCLUSION

Thin layer chromatography is rapid and low-cost technique for screening investigation of plant metabolites, however the similarity of isoflavones chemical structure causes the difficulties of their separation. The best separation all tested compounds was obtained on RP-18s plates with use the mixture of diisopropyl ether/toluene/formic acid 40/60/0.5 v/v/v as a mobile phase. Presented chromatographic system can be useful for qualitative analysis of isoflavones plant material.

**Table 1: $hR_F$ values for investigated compounds obtained with use mobile phases according to literature**

| Mobile phase composition | coumestrol | formononetin | daidzein | genistein | prunetin | biochanin A |
|---|---|---|---|---|---|---|
| chloroform/methanol/water 23/8/1 v/v/v [13] | 60.8 | 65.7 | 58.4 | 60.2 | 66.9 | 67.5 |
| dichloromethane/ ethyl acetate /acetic acid 12/1/2 v/v/v [14] | 38.9 | 51.8 | 27.5 | 39.6 | 63.2 | 63.5 |
| chloroform/methanol/ ethyl acetate / water 16.2/18.8/52/3 v/v/v [15] | 73.9 | 75.2 | 72.7 | 74.6 | 77.7 | 78.4 |

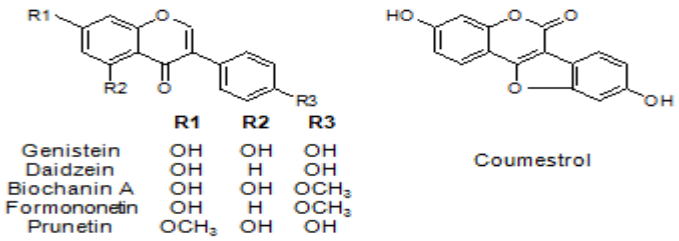


**Fig. 1: Chemical structures of investigated compounds**

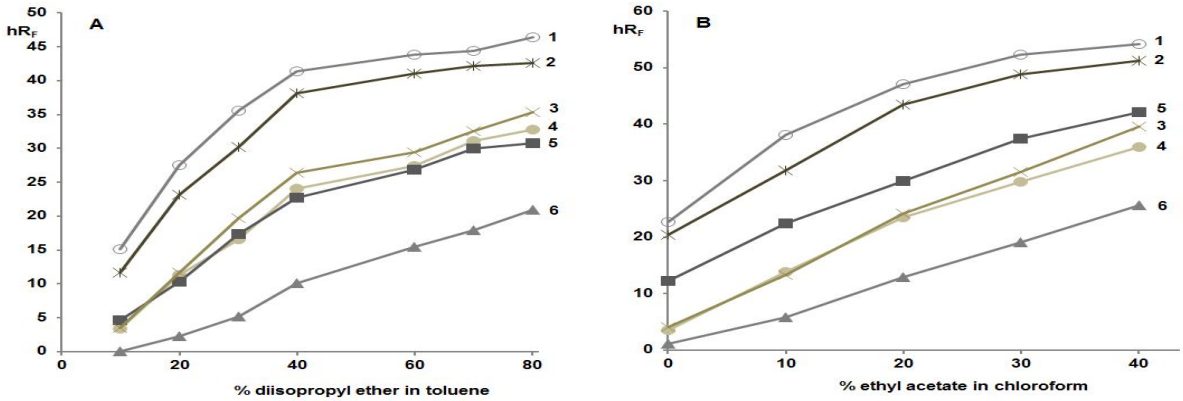


**Fig. 2: Relationship between $hR_F$ values and amount (%) of diisopropyl ether in toluene (A) or ethyl acetate in chloroform (B) on HPTLC Si 60. Standards: 1- biochanin A, 2- prunetin, 3- genistein, 4- coumestrol, 5- formononetin, 6- daidzein.**

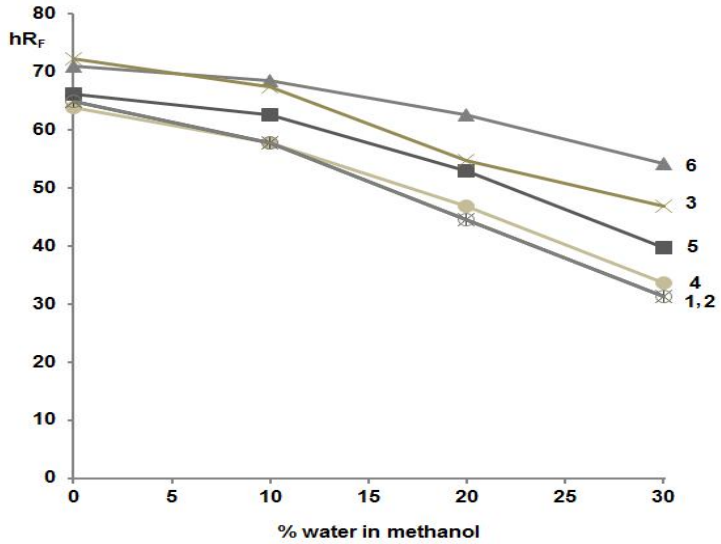


**Fig. 3: Selectivity of isoflavones separation on: aminopropyl silica ( I – 30% water in methanol, II – 20% water in acetonitrile, III – 10% methanol in chloroform) and cyanopropyl silica ( IV – 10% ethyl acetate in chloroform, V – 20% ethyl acetate in toluene, VI – 30 % diisopropyl ether in toluene, VII – 30 % water in methanol). Standards: 1- biochanin A, 2- prunetin, 3- genistein, 4- coumestrol, 5- formononetin, 6- daidzein**

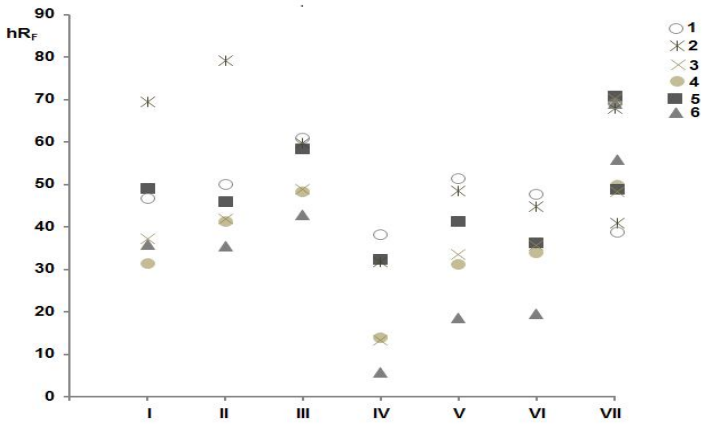


**Fig. 4: Relationship between $hR_F$ values and amount (%) of water in methanol on HPTLC RP-$18_W$. Standards: 1- biochanin A, 2- prunetin, 3- genistein, 4- coumestrol, 5- formononetin, 6- daidzein**

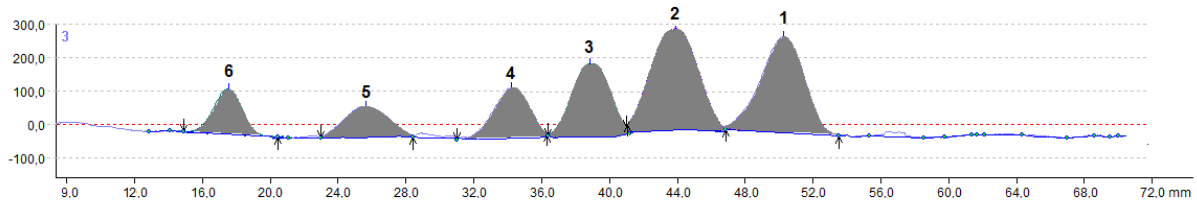


**Fig. 5: The densitogram of standard mixture obtained at λ=260 nm on RP-$18_S$ plates with use diisopropyl ether/toluene/formic acid (40/60/0.5 v/v) as a mobile phase. Standards: 1- biochanin A, 2- prunetin, 3- genistein, 4- coumestrol, 5- formononetin, 6- daidzein.**

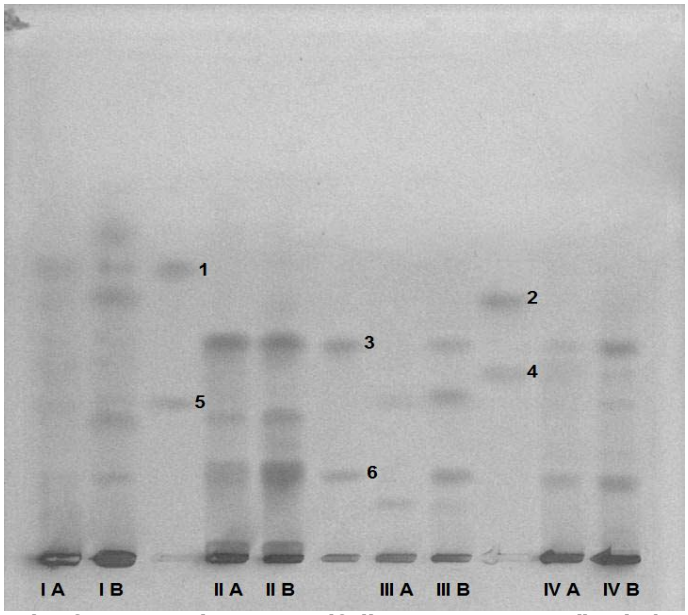


**Fig. 6: The photograph of HPTLC plate: I – *Trifolium pratense* L. *(herba)*; II – *Genista tinctoria (herba);* III- *Glycine max (semen)* IV- *Glycine max (herba)* (A-before, B-after hydrolysis). Standards: 1- biochanin A, 2- prunetin, 3- genistein, 4- coumestrol, 5- formononetin, 6- daidzein. Chromatographic condition: RP-18$_s$ plates with use diisopropyl ether/toluene/formic acid (40/60/0.5 v/v/v) as a mobile phase**

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