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## ANALYSIS OF FLAVONOIDS CONTENT IN ALFALFA

### ANALIZA ZAWARTOŚCI FLAWONOIDÓW W LUCERNIE

**Abstract:** Flavonoid glycosides constitute important group of plant secondary metabolites. This class of natural products play significant role in different physiological processes. Liquid chromatography (HPLC) was used to determine the flavonoid profiles and their concentration in aerial parts of three alfalfa (*Medicago sativa* L.) cultivars. It was shown that flavonoids of alfalfa are glycosides of four flavone aglycones: apigenin, luteolin, triclin and chrysoeriol. All flavonoid glycosides possessed glucuronid acid in sugar chain. Some of them were acylated with ferulic, coumaric or sinapic acids. It was shown that dominant flavonoids of alfalfa were the flavones: triclin and apigenin glycosides (65–72 % of total). The concentration of luteolin and chrysoeriol glycosides did not exceed 30 % of the total. The dominant flavonoid in Radius and Sapko cultivars was glucoside of triclin, in Sitel cultivar glycoside of apigenin. Thus, triclin and apigenin glycosides were the major flavones found in alfalfa aerial parts.

**Keywords:** *Medicago sativa*, flavonoids, herbivore, *Acyrtosiphon pisum*

Alfalfa (*Medicago sativa* L. (Fabaceae)) is the world's oldest and most important livestock feeding crop [1]. Alfalfa has great potential as food and/ or fodder. High nutritional quality of alfalfa has been determined by the high content of good quality protein and carbohydrates [2, 3]. Alfalfas are one of sources of chlorophyll, vitamins, some digesting enzymes and  $\beta$ -carotene [4]. However, alfalfa contains different classes of secondary metabolites, showing biological activities, but they are not yet fully characterized. The best recognized secondary metabolite groups are carotenoids and saponins [5, 6]. In alfalfa the phenolic composition, including flavonoids is poorly understood [7].

Flavonoids are the group of secondary metabolites that can be found in most of the plant families. Most flavonoids in plant cells are present as glycosides. Flavonoid glycosides constitute a structurally diverse group of plant secondary metabolites [8].

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The flavonoids are usually divided into many subclasses: flavones, isoflavones, flavanones, anthocyanins, aurones and others. These natural products play a very important role in plant development and physiology, especially during their interactions with other living organisms [9]. Flavonoid glycosides and free aglycones are involved in pathogenic and symbiotic interactions with microorganisms [10, 11]. They also act as UV protectants in plant cells [12], pigment sources for flower colouring compounds [13, 14] and they play important roles in interactions with insects [15]. This class of compounds also affect the human and animal health because of their significance in the diet, which is ascribed to their antioxidant and UV properties [16–19], estrogenic action [20] and a wide spectrum of antimicrobial and pharmacological activities [21, 22].

Recent work on alfalfa flavonoids revealed that they consist of apigenin, luteolin, triclin and chrysoeriol glycosides and the only sugar unit found in sugar chains is glucuronic acid. It was documented that a number of flavones are acylated with caffeic, ferulic or sinapic acids [23–25]. Very little is, however, known about the concentration of flavones in alfalfa cultivars. Due to the importance of the flavonoids and their glycosides too and in living organisms, the identification and determination the level of such compounds occurring in plant tissue or other biological systems play an important role in many areas of science, particularly in plant science. Thus, the aim of the present study was to analyse the flavonoid profiles and to determine their concentration in green aerial parts of three alfalfa cultivars which are common crops in Poland.

## Material and methods

Alfalfa (*Medicago sativa* L.) (Fabaceae), Radius, Sapko and Sitel, was used in the experiments. Cultivars of alfalfa, Radius and Sapko, were obtained from Institute of Plant Breeding and Acclimatisation (IHAR), Radzikow/ Blonie, near Warsaw. Cultivar Sitel was bought in Horticultural Plant Breeding, Seed Production and Nursery in Ozarow Mazowiecki, Poland. Seeds of the studied cultivars were germinated in a climatic chamber kept at  $21 \pm 1$  °C, L16:D8 photoperiod, and 70 % r.h. The plants were grown in  $7 \times 7 \times 9$  cm plastic pots in a standardised soil mixture structure, one plant per pot. The plants were regularly watered and no extra fertiliser was added. The six months old plants of alfalfa were used in the experiments.

Aerial parts of the 6-month-old plants were harvested, freeze-dried, ground, and kept in a desiccator in darkness until analysed. Each extracts were obtained using the ASE 200 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, USA) for 20 minutes with 70 % methanol. The extracts were concentrated at 40 °C on a rotary evaporator until the methanol was removed and then loaded on C<sub>18</sub> cartridges (Waters, Poland) preconditioned with water. The flavonoids were then successively washed from the cartridges with water and 40 % methanol. Eluates were evaporated to the dryness, redissolved in 1 cm<sup>3</sup> of 40 % MeOH and used for HPLC determination.

The high performance liquid chromatographic (HPLC) analysis was performed on a Waters Alliance liquid chromatograph (Waters, Milford, MA) equipped with a model 616 pump, a model 600s controller, and a model 996 photodiode array detector. The

Millenium Chromatography Manager was used to monitor chromatographic parameters and to process the data. The alfalfa samples were applied to a Eurospher PD 82 column and eluted at  $1 \text{ cm}^3 \text{ min}^{-1}$  with a linear gradient of 1 % phosphoric acid in water : 40 % acetonitrile in 1 %  $\text{H}_3\text{PO}_4$  (65:35 %), increasing to 0:100 % over 60 min. Chromatograms were registered and integrated at  $\lambda = 350 \text{ nm}$ . Standards of glycosides, purchased from the Biochemical Laboratory Institute of Soil Science and Plant Cultivation (Pulawy, Poland), were used for calibration curve preparation.

## Results and discussion

Flavonoid glycosides are present in the plants. They have great diversity, which suggest their function in plant – herbivores association (oviposition, feeding stimulants, toxicity and inhibitory effect for insects) [16]. Because Fabaceae are important crops it seems crucial to characterize its flavonoid composition and concentration.

The HPLC flavonoid profiles of studied cultivars are similar and contain eighteen individual compounds (Fig. 1). These compounds showed absorption spectra characteristic for apigenin (six glycosides), luteolin (three glycosides), chrysoeriol (one glycoside) and tricrin (eight glycosides) derivatives [23–25].

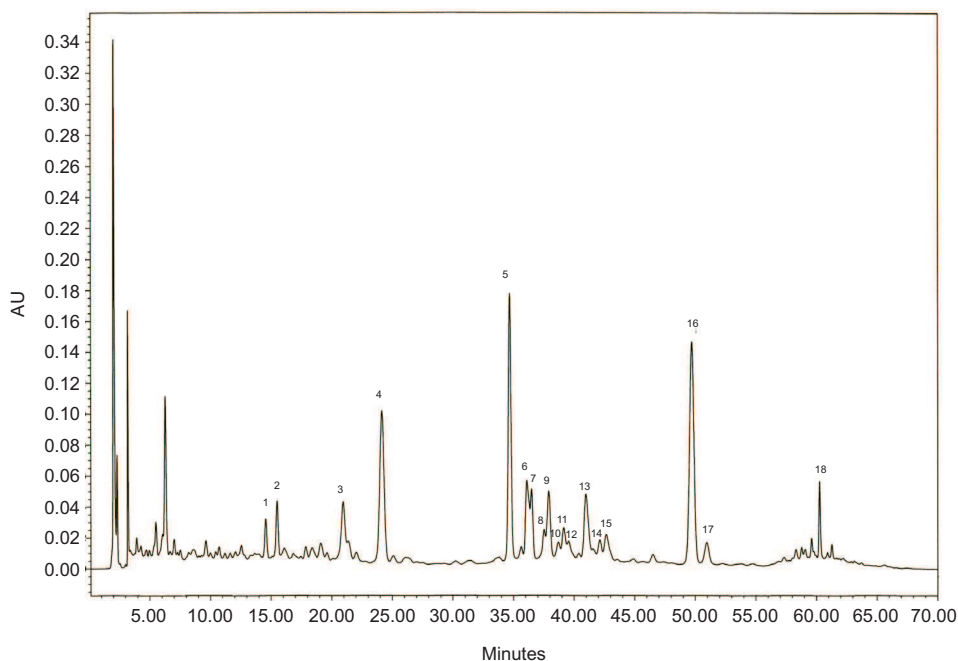


Fig. 1. Identified flavone aglycones: (1) apigenin; (2) luteolin; (3) luteolin; (4) luteolin – acylated; (5) apigenin – acylated; (6) chrysoeriol; (7) apigenin – acylated; (8) apigenin – acylated; (9) apigenin; (10) tricrin; (11) tricrin – acylated; (12) tricrin – acylated; (13) apigenin – acylated; (14) tricrin; (15) tricrin; (16) tricrin; (17) tricrin; (18) tricrin

The structures of ten individual flavones were elucidated with MS method (Fig. 2). These compounds have been previously separated from alfalfa aerial parts and their structures were confirmed by UV, MS and NMR spectroscopy [23–25]. All flavonoid glycosides possessed glucuronic acid in sugar chain. Some of them were acylated with ferulic, coumaric or sinapic acids. Compounds 4, 5, 7, 12 and 13 were acylated with ferulic, compound 8 with coumaric and compound 11 with sinapic acids.

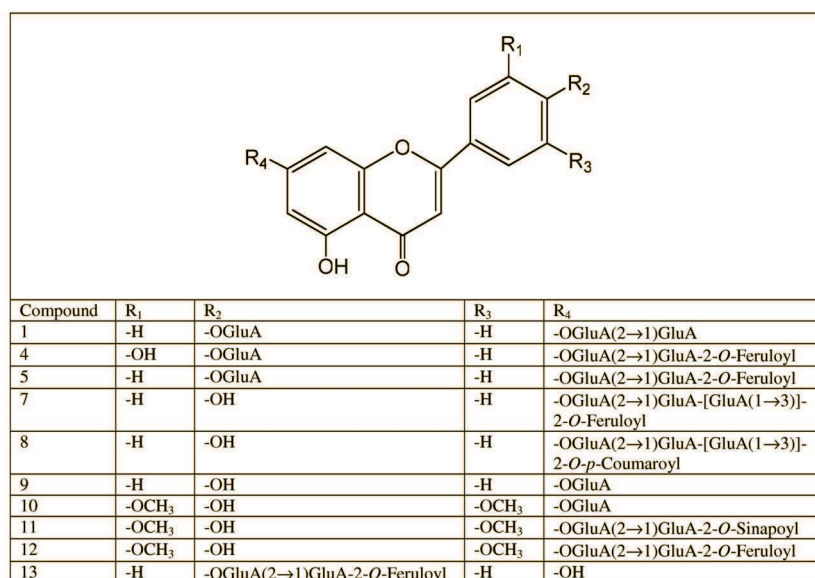


Fig. 2. Chemical formula of analyzed alfalfa flavones

Alfalfa cultivars demonstrated differences in concentration of the individual flavone glycosides (ANOVA,  $p < 0.001$ ) (Fig. 3). The major flavones found in alfalfa aerial parts were tricrin and apigenin glycosides (66–72 % of total). The flavonoids

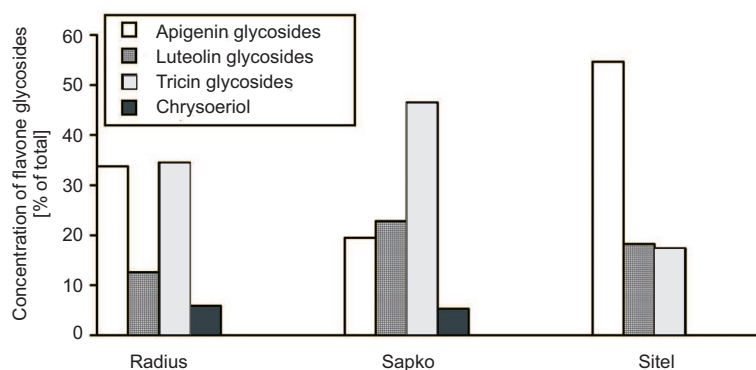


Fig. 3. Content of flavone glycosides from aerial parts of alfalfa cultivars

concentration within alfalfa aerial parts was rather high as compared with other plant sources [18, 26]. For Radius cultivar the tricetin glycosides were the dominant and made up 34.5 % of total flavonoids. Their concentration was very similar to the sum of apigenin glycosides (33.8 % of total). Luteolin and chrysoeriol made up 12.6 and 5.9 % of total, respectively. For Sapko cultivar the tricetin glycosides were the dominant, too and made up 46.5 % of total. Apigenin, chrysoeriol and luteolin made up 19.5, 5.3 and 22.8 % of total, respectively. For Sitel cultivar the apigenin glycosides were the dominant (54.6 % of total). Concentration of tricetin, chrysoeriol and luteolin did not exceed 36 % of total. Thus, tricetin and apigenin glycosides were the major flavones found in alfalfa aerial parts. Stochmal and Oleszek [26] made similar observations.

Flavonoid analyses revealed a substantial individual variation (ANOVA,  $p < 0.001$ ). It was shown that compounds number 5 and 16 were the dominant apigenin and tricetin glycosides of alfalfa cultivars (Fig. 4). Stochmal and Oleszek [26] showed that in all tested varieties the dominant flavonoid was tricetin glycosides.

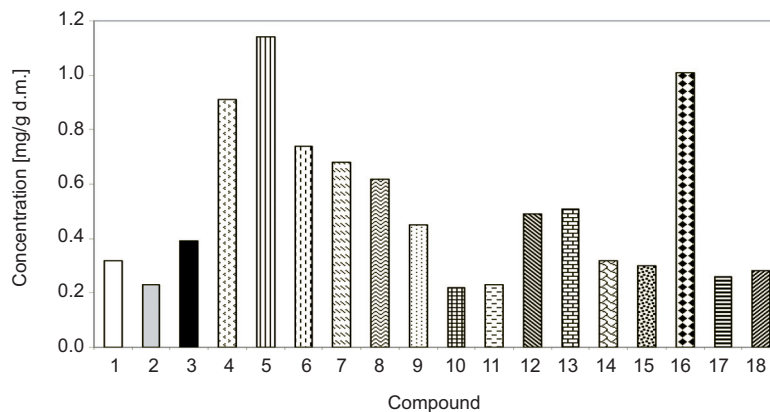


Fig. 4. The concentration of individual flavones from aerial parts of alfalfa cultivars (average for three studied alfalfa cultivars)

Alfalfa flavones are a mixture of acylated and nonacylated forms. The ratio of acylated to nonacylated forms in the plants are important, because the concentration of these compounds probably may reflect the influence of environmental conditions [27]. Considering this fact, accumulation of acylated and nonacylated forms in different cultivars were also analyzed. The average total concentration of acylated flavones in studied alfalfa cultivars ranged between 3.77–5.58 mg/g d.m. There was no significant difference in the concentration of acylated and nonacylated flavones between studied cultivars. The ratio of acylated to nonacylated flavones for studied alfalfa cultivars was similar, too (0.89–1.14).

## Conclusions

1. It was shown that the HPLC flavonoid profiles of studied cultivars are similar.

2. Flavonoids of alfalfa are glycosides of four flavone aglycones: apigenin, luteolin, tricetin and chrysoeriol.

3. Dominant flavonoids of studied alfalfa were the flavones: tricetin and apigenin glycosides (65–72 % of total). The concentration of luteolin and chrysoeriol glycosides did not exceed 30 % of the total.

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## ANALIZA ZAWARTOŚCI FLAWONOIDÓW W LUCERNIE

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**Abstrakt:** Wobec doniesień literaturowych ukazujących różne aspekty interakcji roślina–owad celowe wydaje się poszukiwanie roślinnych substancji chemicznych biorących udział w obronie roślin przed negatywnym wpływem biotycznych czynników środowiskowych. Przykładowo, flawonoidy oddziałują jako deterynty pokarmowe, inhibitory trawienia, a także bezpośrednie toksykanty w stosunku do owadów. Lucerna z uwagi na bogactwo struktur flawonów jest dobrym modelem do prowadzenia prac związanych z tym zagadnieniem. Wiedza na temat występowania flawonoidów w lucernie siewnej, będącej jedną z głównych roślin pastewnych, nie jest pełna, dlatego celem przeprowadzonych badań było zidentyfikowanie i porównanie zawartości flawonoidów w powszechnie użytkowanych odmianach lucerny siewnej (*Medicago sativa* L.).

Badaniom poddano trzy odmiany lucerny: Radius, Sapko i Siteł. Ekstarkcję flawonoidów przeprowadzono z 6-miesięcznych roślin za pomocą metanolu. Rozdział i identyfikację flawonoidów wykonano metodą wysokosprawną chromatografię cieczową (HPLC) sprzężoną ze spektrometrią mas.

Stwierdzono, że flawonoidy badanych odmian lucerny to glikozydy czterech aglikonów flawonów: apigeniny, luteoliny, trycyny i chryzoeriolu. W łańcuchu cukrowym wszystkich glikozydów występował kwas glukuronowy, a niektóre z nich były acylowane kwasem ferulowym, kumarowym bądź synapinowym. Wykazano, że dominującymi związkami były pochodne trycyny i apigeniny. Łączna zawartość glikozydów apigeniny i trycyny wahała się w granicach 65 do 72 % sumy flawonów badanych lucern. Zawartość glikozydów luteoliny i chryzoeriolu nie przekraczała 30 % sumy flawonów. Dla odmian Radius i Sapko związkiem dominującym był glikozyd trycyny, którego maksymalne stężenie (2.49 mg/ g s.m.) odnotowano w tkankach odmiany Radius. W przypadku odmiany Siteł w największych ilościach występował glikozyd apigeniny (1.31 mg/ g s.m.).

**Słowa kluczowe:** *Medicago sativa*, flawonoid, owady roślinożerne, *Acyrtosiphon pisum*