

# Isoflavones profiles of red clover (*Trifolium pratense* L.) at different growth stages

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## Introduction

Phytoestrogens are plant compounds that can exhibit estrogen-like activity. Isoflavones are one of the best studied groups of phytoestrogens known for their human health benefits. Red clover (*Trifolium pratense* L.) is one of the leading forage legumes in northern and south-eastern Europe, the United States and Canada. In recent years, the use of natural antioxidants found in red clover has attracted interest due to their presumed nutritional and therapeutic values (Vlaisavljevic et al., 2014; Vlaisavljevic et al., 2017). Studies of utilization of red clover by the pharmaceutical industry have pointed that dietary phytoestrogens play an important role in the prevention of menopausal symptoms (Lipovac et al., 2010), osteoporosis (Atkinson et al., 2004a), estrogen-related cancers like breast cancer, prostate cancer (Atkinson et al., 2004b; Velentzis et al., 2008) and heart disease (Cano et al., 2010; Dixon, 2004). The isoflavones content in red clover is controlled by genetic and environmental factors (Boot et al., 2006; Sivesind and Seguin, 2005; Tsao et al., 2006; Visnevschi-Necrasov et al., 2013). The aim of this study was to determine if the isoflavones quantitative and qualitative content depends on different growth stages in red clover variety *Una*.

## Materials and methods

### Sample preparation

Red clover herbage samples (*Una* variety) were collected from four different growth stages (1. bud emergency, 2. first flower stalk emergence, 3. mid of flowering and 4. full flowering) at the experimental field of the Institute of Field and Vegetable Crops, Bački Petrovac, in 2019. Herbage samples were dried at 60 °C for about 48 hours, homogenized and grounded to a particle size of  $\bar{A} = 0.8$  mm, as well as mixed with 2 ml of water at 37 °C. After that, HCl and ethanol were added and this mixture was heated to boiling. Obtained extracts were purified by solid phase extraction on HLB cartridges and analyzed on HPLC after filtration.

### HPLC analysis of isoflavones

Zorbax SB C<sub>18</sub> reversed phase HPLC column was used for separation of main isoflavones present in red clover: formononetin, biochanin A, genistein, and daidzein. Isoflavones were identified by comparing the retention times in HPLC chromatograms and UV spectral patterns with those of standard compounds. Isoflavone concentrations were quantified by external standard method using five-point regression curves of formononetin, biochanin A, genistein, and daidzein standard compounds.

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## Results and discussion

Results of isoflavones analyses in different growth stages of red clover variety *Una* revealed the highest total content in the stage of bud emergency, due to greater proportion of leaves in the samples. In this stage the highest value of two isoflavones content: formononetin (3.21 mg g<sup>-1</sup>) and biochanin A (3.19 mg g<sup>-1</sup>) was found. Moreover, concentrations of formononetin and biochanin A decreased markedly as flowering progressed, while a smaller decrease, found for daidzein and genistein. Previous studies have confirmed the existence of differences in isoflavone content depending on growth stage and it has been noticed that two isoflavones: formononetin and biochanin A, are the most abundant (Booth et al., 2006; Sivesind and Seguin, 2005; Tsao et al., 2006; Lemežienė et al., 2015).

## Conclusion

Red clover variety *Una* harvested at the lowest growth stage can be used as row material for production of dietary supplements due to the significant content of isoflavones.

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