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Phytochemical composition of temperate perennial legumes

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SUPPLEMENTARY MATERIALS

Qualitative characterisation of isoflavone composition

Method. Qualitative characterization of the isoflavone composition of hydrolysed legume extracts was done on Acquity UPLC system (Waters, Milford, MA, USA) using dual-wavelength ultraviolet/visible detector (UV) and compounds were identified as described in our recent work (Taujenis et al. 2015[1]).

Results. The UPLC-UV chromatograms of hydrolysed extracts of selected flowering legumes are presented in Figure S1. Peaks of the target analytes marked in the chromatograms show that besides daidzein, genistein, formononetin and biochanin A, some additional isoflavones (pratensein, irilone and prunetin (retention times 11.93, 12.87 and 13.82 min, respectively), were identified in the extracts of flowering red clover plants of 2014 harvest year from the current experiment and described in our recent work (Taujenis et al. 2015). Pratensein, irilone and prunetin were not quantified in the current study.

Qualitative analysis of UPLC-UV chromatograms of the extracts of alfalfa and especially sainfoin and milkvetch showed (Fig. S1) that the species contain compounds, eluting between daidzein (peak 1) and genistein (peak 2). Their peaks were more intense than those of four quantified isoflavones in these species; however, the compounds were not identified in the current study. Chromatograms of sainfoin and milkvetch extracts were distinguished by intense peaks eluting between genistein (peak 2) and formononetin (peak 3).

[1] Taujenis L, Padarauskas A, Mikaliūnienė J, Cesevičienė J, Lemežienė N, Butkutė B (2015) Identification of isoflavones and their conjugates in red clover by liquid chromatography coupled with DAD and MS detectors. *Chemija* **26**, 107-112.

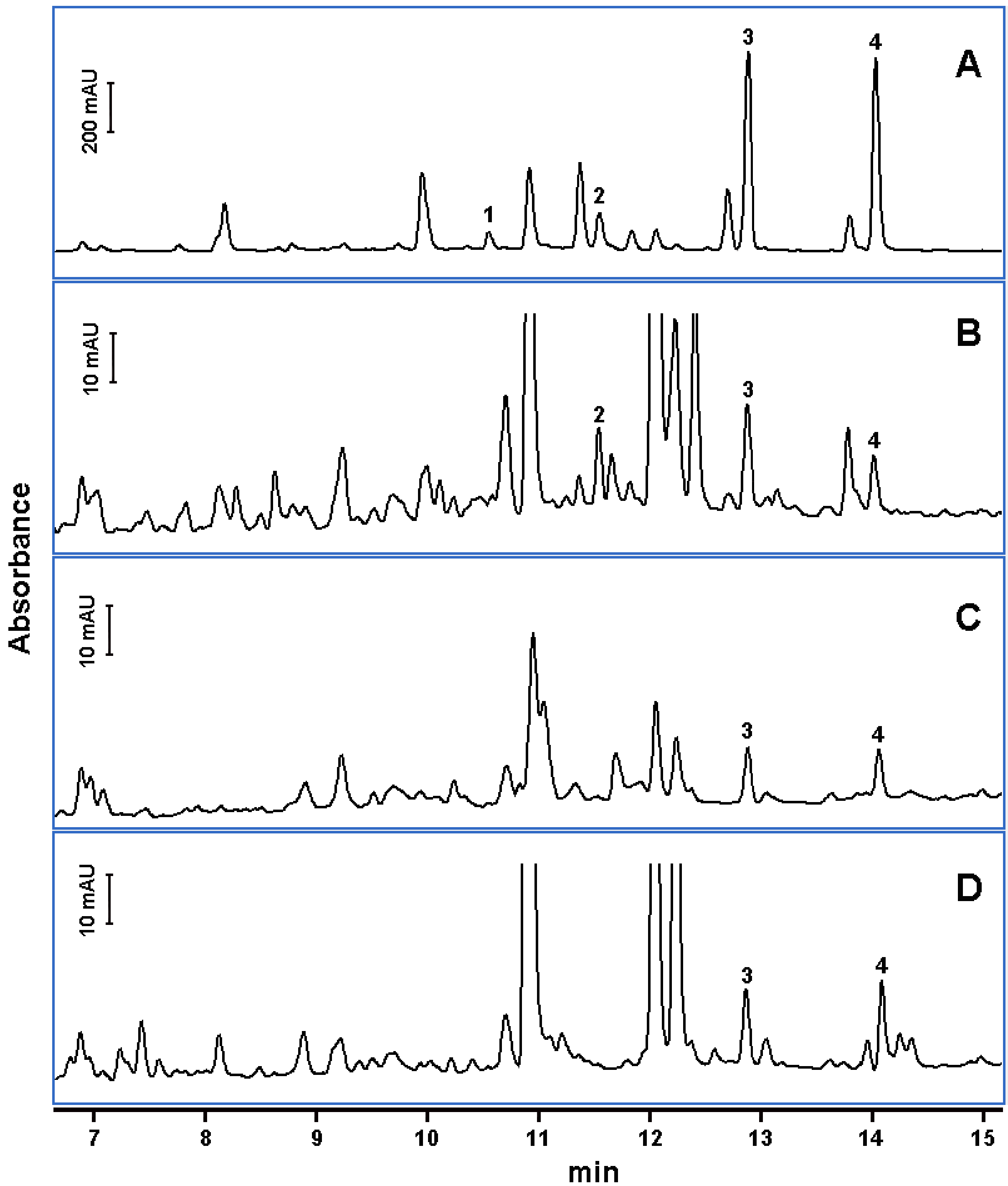

Fig. S1. UPLC-UV chromatograms of the extracts of whole aerial part of flowering legume plants harvested in 2014. A – *T. pratense*; B – *A. glycyphyllos*; C – *M. sativa*; D – *O. viciifolia*. Peaks: 1 – daidzein; 2 – genistein; 3 – formononetin, 4 – biochanin A.

Proximate composition

Methods. Samples were analyzed for crude protein, crude fat, neutral detergent fibre (NDF), and crude ash contents. Crude protein (CP) was determined by the Kjeldahl method with a conversion factor of 6.25; crude fat content was measured gravimetrically by the continuous Soxhlet extraction with hexane. NDF extraction was done on an ANKOM220 Fibre Analyzer (ANKOM Technology, Macedon, NY, USA, ANKOM method 08-16-06) using F57 filter bags (25-µm porosity). The data of NDF are presented as ash-free. Crude ash content was determined by the mass left after sample incineration at 550±10°C. Plant materials were also subjected to analyses of nonstructural carbohydrates (water soluble sugars and starch). Concentrations of soluble sugars in 40% ethanolic extracts were measured spectrophotometrically (M107, Camspec, UK) using the anthrone reagent (Zhao et al. 2010[2]). Starch was determined in plant material residue after soluble sugar washing. The remaining plant material was solubilized and hydrolyzed to glucose using enzymes α-amylase and amyloglucosidase and released glucose was assayed following the general procedures described by Zhao et al. (2010). Data of proximate analysis were expressed in g/kg DM. To determine whether the differences among the nine accessions for each proximate component in sample groups of the same harvest year, growth stage and plant part were significant, one-way analyses of variance (ANOVAs) were used followed by Fisher's least significant difference (LSD) tests. P values <0.05 were considered significant.

Results. The legumes species differed significantly ($P<0.05$) in contents of most nutritional components for both fully flowering and young plants (Table S1). At flowering, the ash content in legume plants ranged from 62.6 to 97.7 g/kg DM, CP content from 127 to 172 g/kg DM, and NDF ranged from 299 to 509 g/kg DM. Flowering plants contained up to 147 g kg^{-1} DM of readily digestible soluble sugars, on a par with starch up to 78.9 g/kg DM. Among fully flowering *Medicago* accessions, forage quality was better for black medick than other medick, i.e. alfalfa cultivars. However, the significance of specific differences in the content of most components

[2] Zhao D, MacKown CT, Starks PJ, Kindiger BK (2010) Rapid analysis of nonstructural carbohydrate components in grass forage using microplate enzymatic assays. *Crop Science* **50**, 1537-1545.

depended on harvest year. When comparing *Trifolium* accessions, zigzag clover contained considerably less NDF, soluble sugars and starch, and more fat and ash than red clover cultivars. The CP concentration in the clover accessions varied inconsistently across the harvest years. Among flowering plants of tested accessions, *A. cicer* exhibited the highest concentration of nonstructural carbohydrates. We detected a lower ash concentration in sainfoin than in other species ($P<0.05$) (both at flowering and in young plants).

As expected, the NDF content of the legumes at stem elongation growth stage was considerably lower than for those at flowering. Leafy young plants had a much higher concentration of CP, crude fat, starch and ash in DM but less soluble sugars than flowering plants. Variation in proximate composition among legumes of stem elongation stage was lower, though significant differences were observed also.

Table S1. The proximate composition of perennial legumes

The different letters (a, b, c, d, e) in the column indicate significant differences ($P < 0.05$) in the respective component concentrations among the legume accessions within the sample group identical for harvest year and growth stage

Legume accession	Component concentration ± SE (g/kg DM)					
	Ash	Protein	Fat	NDF	Soluble sugars	Starch
			2013, full flowering stage			
T. pratense 'Sadūnai'	83.4±0.8 bc	153±0.4 ab	25.2±0.1 c	374±2.0 bc	123±2.1 bc	47.4±0.2 bc
T. pratense 'Vyčiai'	90.2±1.8 abc	166±2.5 a	34.1±0.2 c	379±5.6 b	105±1.8 cde	30.4±0.3 ef
T. medium	95.6±1.6 a	150±2.1 ab	44.4±0.3 a	336±6.1 cde	88.2±1.2 e	26.3±0.8 f
M. sativa 'Malvina'	82.4±0.7 cd	152±2.9 ab	27.2±0.9 c	439±3.6 a	95.7±1.3 de	36.1±0.8 de
M. sativa 'Birutė'	83.6±0.8 bc	161±2.5 a	29.5±0.6 c	425±7.6 a	98.7±1.8 de	43.0±0.9 cd
M. lupulina 'Arka'	71.5±1.1 de	165±2.3 a	31.9±0.6 bc	376±6.8 bc	128±0.9 ab	52.4±0.9 b
O. viciifolia 'Meduviai'	62.6±1.0 e	133±2.4 c	26.0±0.8 c	349±0.1 bcd	88.4±1.1 e	33.8±0.2 e
A. glycyphyllos	94.2±1.2 ab	156±2.3 ab	39.9±1.0 ab	320±7.2 de	117±1.7 bcd	35.2±0.1 e
A. cicer	84.9±1.7 abc	143±0.4 bc	28.4±0.9 c	299±5.5 e	147±2.1 a	78.9±1.2 a
			2014, full flowering stage			
T. pratense 'Sadūnai'	71.6±0.6 cd	127±2.3 d	25.1±0.8 d	427±3.0 c	136±2.3 a	47.9±0.2 ab
T. pratense 'Vyčiai'	85.0±1.6 b	149±3.1 bcd	33.6±0.2 a-d	384±2.5 cd	120±2.2 ab	29.6±0.3 bc
T. medium	97.7±1.4 a	167±2.5 ab	41.2±0.9 a	359±5.7 d	67.9±1.6 e	20.7±0.6 c
M. sativa 'Malvina'	80.0±1.0 bc	147±2.3 bcd	29.4±0.9 cd	482±6.4 ab	79.8±1.2 de	28.6±0.8 bc
M. sativa 'Birutė'	79.4±1.6 bc	145±2.1 bcd	26.6±1.0 d	509±7.1 a	78.7±0.9 de	30.6±0.8 bc
M. lupulina 'Arka'	83.3±1.2 b	172±0.8 a	39.3±1.0 ab	385±1.0 cd	93.4±0.8 cd	37.6±0.6 abc
O. viciifolia 'Meduviai'	62.4±0.8 d	152±1.2 bc	32.2±0.9 bcd	401±6.0 cd	131±1.6 ab	36.6±0.2 abc
A. glycyphyllos	76.6±1.1 bc	131±1.7 cd	36.9±1.0 abc	380±1.4 cd	114±1.7 bc	26.4±1.3 c
A. cicer	80.8±1.2 bc	156±0.4 ab	31.4±0.6 bcd	432±4.3 bc	111.7±1.9 bc	52.6±1.4 a
			2014, stem elongation stage			
T. pratense 'Sadūnai'	111±2.1 a	231±1.2 b	55.8±0.8 ab	316±4.3 a	64.2±1.2 b	50.8±1.2 cd
T. pratense 'Vyčiai'	114±1.3 a	239±2.0 ab	58.8±1.2 a	325±4.8 a	75.0±0.9 ab	47.0±1.0 cde
T. medium	104±1.8 a	190±4.5 c	42.6±0.1 e	309±2.3 a	79.0±1.5 ab	39.7±0.8 de
M. sativa 'Malvina'	107±1.6 a	246±0.0 a	48.3±0.6 cd	309±2.1 a	75.9±0.7 ab	60.7±1.2 bc
M. sativa 'Birutė'	110±1.1 a	252±3.7 a	47.5±0.0 cd	308±5.6 a	74.3±1.8 ab	50.0±1.1 cd

M. lupulina 'Arka'	102±1.0 a	237±2.4 ab	55.3±0.5 abc	309±4.2 a	99.0±1.4 a	82.1±2.0 a
O. viciifolia 'Meduviai'	80.4±1.3 b	205±1.2 c	46.7±0.5 de	278±3.5 ab	84.1±1.2 ab	34.4±0.4 e
A. glycyphyllos	108±1.7 a	227±3.0 b	51.8±0.3 bcd	278±3.1 ab	83.1±1.0 ab	60.3±1.7 bc
A. cicer	112±1.6 a	249±4.3 a	49.1±0.9 bcd	232±2.7 b	78.6±1.1 ab	73.2±1.2 ab

The accession-dependent differences ($P<0.05$) in the concentration of proximate components were determined for all plant parts of the flowering legumes (Table S2); however, the character of variation in component concentrations differed between the plant parts. For instance, stems of *T. pratense* 'Sadūnai' and *A. cicer* accumulated significantly ($P<0.05$) more soluble sugars than other legumes, while flowers of alfalfa cultivars and leaves of *Astragalus* species and *M. sativa* 'Malvina' were the richest in sugar ($P<0.05$) in comparison with a respective plant part of other legumes.

Table S2. The proximate composition of morphological plant parts of perennial legumes at full flowering stage

The different letters (a, b, c, d, e, f and g) in the column indicate significant differences (P<0.05) among the legume accessions for the respective nutritional component concentration within each plant part separately.

	Component concentration ± SE (g/kg DM)					
Legume accession	Ash	Protein	Fat	NDF	Soluble sugars	Starch
Stems						
T. pratense 'Sadūnai'	48.0±0.8 de	66.2±0.1 d	22.7±0.9 a	486±6.8 c	191±2.8 a	51.0±3.1 a
T. pratense 'Vyčiai'	55.6±0.5 c	74.2±0.8 cd	16.2±0.3 ab	476±4.6 c	157±2.9 c	23.9±2.6 b
T. medium	89.8±0.7 a	96.8±1.2 ab	12.6±0.5 ab	447±6.5 d	69.1±2.7 f	13.5±1.3 cd
M. sativa 'Malvina'	48.9±0.8 d	86.5±2.1 bc	10.4±0.1 b	653±3.3 a	97.6±2.9 e	11.7±1.0 cd
M. sativa 'Birutė'	45.0±0.4 ef	96.6±0.9 ab	12.1±0.2 ab	630±4.1 a	94.8±2.4 e	11.0±0.3 d
M. lupulina 'Arka'	62.3±0.1 b	103±2.1 a	14.1±0.4 ab	543±3.8 b	125±0.6 d	13.4±0.5 cd
O. viciifolia 'Meduviai'	41.9±0.6 fg	72.9±1.7 cd	12.2±0.6 ab	553±2.4 b	167±0.5 bc	12.8±0.8 cd
A. glycyphyllos	41.1±0.1 g	69.4±2.5 d	20.9±0.1 a	559±4.9 b	179±2.6 ab	20.0±0.8 bc
A. cicer	42.3±0.2 fg	71.2±4.1 d	13.3±0.7 ab	535±3.3 b	185±0.5 a	14.7±0.3 cd
Leaves						
T. pratense 'Sadūnai'	100±2.5 b	217±3.7 de	57.2±0.6 c	302±5.1 b	92.6±3.5 bc	32.0±1.2 ab
T. pratense 'Vyčiai'	101±0.6 b	236±0.8 ab	63.4±0.2 b	315±7.1 b	52.8±0.1 e	34.5±1.3 ab
T. medium	98.0±2.1 bc	219±0.4 cde	69.0±0.4 a	348±3.3 a	53.2±2.8 e	35.4±1.7 a
M. sativa 'Malvina'	103±1.6 b	234±1.6 abc	59.0±0.4 c	327±4.9 ab	101±5.0 abc	25.5±1.0 bcd
M. sativa 'Birutė'	115±1.9 a	233±0.8 abc	59.2±0.6 c	355±6.4 a	69.8±0.8 de	26.1±1.9 bcd
M. lupulina 'Arka'	88.3±0.5 d	249±5.1 a	59.5±0.3 c	350±4.9 a	81.5±0.9 cd	29.9±0.1 abc
O. viciifolia 'Meduviai'	76.4±0.6 e	231±1.2 bcd	65.0±0.8 b	257±2.8 c	85.9±3.6 cd	18.3±0.1 d
A. glycyphyllos	89.7±1.3 cd	177±1.2 f	38.8±0.9 d	273±5.5 c	113±3.6 ab	22.5±2.7 cd
A. cicer	98.1±0.6 b	210±5.3 e	35.9±0.8 d	308±4.1 b	119±1.6 a	22.3±2.3 cd
Flowers						
T. pratense 'Sadūnai'	67.8±0.6 de	193±0.0 b	28.1±0.8 b	346±1.0 ab	87.4±1.6 d	18.1±0.8 c
T. pratense 'Vyčiai'	74.2±0.2 bc	185±5.3 bc	22.4±0.3 b	342±2.0 ab	81.8±0.8 d	19.1±0.7 bc
T. medium	72.9±1.2 cd	176±0.0 c	23.3±0.9 b	376±7.9 a	85.9±0.8 d	19.5±0.3 bc
M. sativa 'Malvina'	63.8±0.6 ef	217±1.8 a	30.3±0.2 ab	241±7.0 c	191±0.9 ab	23.6±0.3 bc

M. sativa 'Birutė'	66.0±0.4 e	227±0.6 a	31.0±0.8 ab	246±6.5 c	197±0.5 a	21.4±2.5 bc
M. lupulina 'Arka'	60.6±0.5 f	202±0.2 b	28.1±1.4 b	256±7.3 c	144±0.2 c	34.7±0.9 a
O. viciifolia 'Meduviai'	67.8±0.1 de	217±0.4 a	44.8±0.9 a	381±9.7 a	88.6±0.7 d	24.8±1.3 b
A. glycyphyllos	78.8±2.1 b	178±1.8 c	29.4±0.3 ab	262±5.7 c	187±0.6 b	18.7±0.1 c
A. cicer	91.8±0.3 a	228±0.8 a	25.6±0.5 b	314±6.2 b	109±0.4 d	18.0±0.5 c

Stems and leaves of alfalfa contained more NDF, while flowers had less NDF than the corresponding morphological fraction of the remaining accessions. Empirically, the differences in the proximate composition among the plant parts were markedly higher than those among the legume species. Due to the higher CP, ash, and fat contents and lower NDF concentrations, proximate composition of leaves was considerably better than that of stems. The CP level in leaves did not drop below 177 g/kg DM, whereas in stems it did not exceed 103 g/kg DM. The CP content in flowers varied within a similar range as in leaves. The differences in ash and fat contents among the plant parts were also very marked for all accessions. The concentrations of these components in plant parts decreased in the following order: leaves > flowers > stems. The NDF content was considerably higher in the stems than in the flowers and leaves.