XIII EQA

PORTO 14-16 SETEMBRO

LIVRO DE ATAS

Livro de Atas do XIII Encontro de Química dos Alimentos

Disponibilidade, valorização e inovação: uma abordagem multidimensional dos alimentos

14 a 16 de setembro de 2016

Porto, Portugal

Universidade do Porto
LAQV/REQUIMTE
Sociedade Portuguesa de Química

Ficha Técnica

Título: Livro de Atas do XIII Encontro de Química dos Alimentos

Autor: Comissão Organizadora

Tipo de suporte: Eletrónico

Detalhe do suporte: PDF

Edição: 1.ª Edição

ISBN: 978-989-8124-15-9

Ano 2016

Esta publicação reúne as comunicações apresentadas no XIII Encontro de Química dos Alimentos sob a forma de ata científica.

A aceitação das comunicações foi feita com base nos resumos apresentados: o texto integral que aqui se reúne é da inteira responsabilidade dos autores.

Effects of phenologic factors on the expression of isoflavones in *Medicago* genus

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Keywords: *Medicago* spp.; phenologic factors; isoflavones

ABSTRACT

There is a high variety of plant species that are often proposed as potential natural sources of specific bioactive components, with emphasis in phenolic compounds. However, the ability to produce a determined phytochemical might vary along their vegetative cycle, even among species with close phylogeny. Herein, the association among isoflavone production and phenologic determinants was verified in nine *Medicago* spp. Isoflavone profiles were characterized in field-grown plants in three phenologic stages (vegetative elongation, VE; late bud, LB; late flowering, LF). Isoflavones were extracted by matrix solid-phase dispersion method and analyzed using high-performance liquid chromatography coupled with a diode-array detector [1]. Formononetin, genistein and irilone were the most abundant isoflavones, reaching values higher than those present in acknowledged plant sources like soy or red clover [2]. Regarding their evolution along the vegetative cycle, it was verified that the isoflavones did not follow the same tendency, being possible to conclude the phenologic stage that optimizes the expression of each single isoflavone. Furthermore, some particular distinctive trends were observed for each analyzed *Medicago* species. In addition, there was a great interaction among each assayed species. Accordingly, using monoculture crops would be a more reliable option to achieve the optimal conditions for harvesting plants with a desirable isoflavone profile. In general, this study promoted *Medicago* spp. as potential isoflavone sources, with potential application as foodstuff, feedstuff, or in the nutraceutical industry.

1. INTRODUCTION

The genus *Medicago* is part of the botanical family of Leguminosae and includes about 56 different species. Alfalfa (*Medicago sativa*) is the mainly grown *Medicago* species

throughout the world, but several other *Medicago* species might have potential as sources of phytochemicals [3]. Isoflavones are known as having a wide range of beneficial biological activities in the human body, but their overconsumption have been suggested as potentially causing adverse effects. Hence, the intake of isoflavones has been limited by International Organisms (such as Food Safety Commission of Japanese Government or The Nutrient Data Laboratory of the Agricultural Research Service of the United States Department of Agriculture) to very restrict values [4]. In fact, the biosynthesis of isoflavones varies greatly with environmental and genotypic factors, but also with the phenological stage, fluctuating along the plant maturity [5]. Furthemore, isoflavones profile is highly affected by genotype×environment interactions [6].

Accordingly, the profiles in free and conjugated isoflavones were compared among open-field grown *Medicago* spp. and the changes along vegetative cycle were monitored by evaluating three different phenologic stages: vegetative elongation, late bud and late flower. With this approach, it was intended to evaluate the effect of the plant species and the phenologic stage (as well as the interaction of both factors) in the potential yield of individual and total isoflavones.

2. MATERIALS AND METHODS

2.1. Plant material and field experimental site

M. arabica, *M. doliata*, *M. minima*, *M. murex*, *M. orbicularis*, *M. polymorpha*, *M. rigidula*, *M. tornata* and *M. truncatula* were sown at the Experimental Field of the University of Porto (Agrarian Station of Vairão). Samples were collected from February to July in three phenologic stages: 1 - vegetative elongation (stem length <30 cm, no visible buds or flowers); 2 - late bud (three or more nodes with visible buds, no flowers or seed pods); and 3 - late flower (one or more nodes with 50% open flowers, no seed pods).

2.2. Extraction procedure and HPLC determination

Matrix solid-phase dispersion (MSPD) extraction of isoflavones was performed following a previous method [1]. Before HPLC analysis, the extracts collected in amber vials were filtered through a 0.45 μm PTFE membrane. Different samples of two distinct accessions of all species were extracted.

Chromatographic analyses were performed following a previously optimized methodology [1]. A high-performance liquid chromatograph (Jasco, Tokyo, Japan) equipped with a PU-2080 quaternary pump and a Jasco AS-950 automatic sampler were used. Detection was performed with a multi-wavelength diode-array detector (DAD) Jasco, MD-2010. Data were analyzed using the Borwin-PDA Controller Software (JMBS, Le Fontanil, France). Compounds were identified by chromatographic comparisons with authentic standards and UV spectra. Quantification was made using the calibration curves obtained for each

identified isoflavone (DAD at 254 nm) based on the internal standard (2-methoxyflavone) method.

2.3. Statistical analysis

All extractions were performed in triplicate and each replicate was quantified twice. Data were expressed as mean±standard deviations All statistical tests were performed at a 5% significance level using the SPSS software, version 22.0 (SPSS Inc).

An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analyzed using 2-way ANOVA, with the factors "plant species" (PSp) and "phenologic stage" (PhS), fixed to evaluate properly the effects of phylogeny and phenology. When a statistically significant interaction (PSp×PhS) was detected, the two factors are evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction is verified, means obtained for each of the assayed factors (PhS or PSp) were compared using Tukey's HSD or Tamhane's T2 tests.

3. RESULTS AND DISCUSSION

In this study, eleven isoflavones were quantified, eluting in the following order: 1) puerarin, 2) daidzin, 3) genistin, 4) daidzein, 5) glycitein, 6) genistein, 7) pratensein, 8) formononetin, 9) irilone, 10) prunetin, 11) biochanin A. **Table 1** shows the isoflavone composition reported as the mean value of each plant species (PSp) considering the three phenologic stage (PhS), as well as mean value of each PhS, individually containing the values for all nine PSp (thereby, the standard deviations should not be understood in *sensu strictu*). The interaction PSp×PhS was significant for all isoflavones, meaning that the variation in isoflavones contents resulted from the conjunct action of both factors simultaneously.

In general, LB and VE presented hugher isoflavones levels, independently of PSp. Likewise, puerarin (*M. doliata* and *M. murex*), daidzin (*M. murex*), genistin (*M. polymorpha*), daidzein (*M. orbicularis* and *M. tornata*), glycitein (*M. doliata*, *M. polymorpha* and *M. truncatula*), pratensein (*M. orbicularis*), formononetin (*M. orbicularis*), prunetin (*M. doliata*), bioachanin A (*M. arabica*, *M. minima* and *M. orbicularis*) were not detected in the LF stage.

The phylogeny seemed to exert the most marked effects on the production of isoflavones in *Medicago* leaves. Independently of the PhS, genistin and biochanin A tended to be present in higher quantity in *M. tornata*, daidzein and prunetin in *M. arabica*, glycitein in *M. murex*, genistein in *M. doliata*, formononetin in *M. orbicularis* and irilone in *M. truncatula*.

M. arabica, *M. doliata* and *M. orbicularis* presented the highest overall content of isoflavones (**Table 1**), which were higher than those presented by other vegetable species like green bean, carrot, white cabbage, cauliflower, iceberg lettuce, artichoke and even soybean [7].

4. CONCLUSION

Medicago species might be considered as an interesting alternative source of isoflavones. The phylogenetic factors induces the greater differences, since the variation along the vegetative cycle was less pronounced. These results are important to use *Medicago* spp. as isoflavone sources, since their effects are highly dependent on type and concentration.

Table 1. Isoflavone contents (mg/kg of dry matter) in the studied *Medicago* species.

		Puerarin	Daidzin	Genistin	Daidzein	Glycitein	Genistein	Pratensein	Formononetin	Irilone	Prunetin	Biochanin A	Quantified isoflavones
Plant species (PSp)	*M. arabica*	1±1	nd	1±1	19±7	nd	104±38	19±5	2010±490	52±22	96±37	40±34	2342±538
	M. doliata	7±10	nd	3±3	7±3	7±5	664±118	nd	1546±395	225±73	7±11	37±10	2502±572
	M. minima	nd	nd	5±2	nd	21±9	42±11	25±6	1158±435	141±30	18±26	28±25	1437±448
	M. murex	22±32	6±8	4±1	9±2	63±10	115±18	4±3	1215±298	64±14	8±3	18±4	1527±279
	M. orbicularis	nd	nd	4±1	1±1	9±13	206±23	21±16	2806±192	103±16	nd	16±13	3166±231
	M. polymorpha	nd	nd	5±4	nd	4±5	390±227	47±5	11±16	1432±272	nd	nd	1889±455
	M. rigidula	nd	nd	38±15	nd	nd	104±51	nd	1185±353	26±18	nd	25±6	1378±378
	M. tornata	26±37	1±2	169±78	3±4	7±4	103±51	3±4	620±219	217±113	38±18	63±36	1250±382
	M. truncatula	nd	nd	15±13	1±1	1±1	440±119	45±23	824±243	696±249	nd	nd	2021±535
	p-value (n = 36)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phenologic stage (PhS)	1 - VE	10±21	2±5	26±43	4±8	11±15	208±172	17±18	1104±779	242±317	16±23	42±36	1682±707
	2 - LB	nd	nd	39±84	5±7	11±19	269±252	22±24	1478±751	390±473	15±34	22±14	2252±596
	3 - LF	9±24	1±1	15±30	4±5	15±25	245±229	16±17	1208±903	354±536	25±41	13±16	1904±790
	p-value (n = 108)	<0.001	<0.001	0.009	0.277	0.234	0.122	0.053	<0.001	0.005	0.058	<0.001	<0.001
PSp×PhS	*p*-value (n = 324)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Acknowledgements

The authors are grateful to Foundation for Science and Technology (FCT, Portugal) for financial support to REQUIMTE (PEst-C/EQB/LA0006/2014) and to CIMO (UID/AGR/00690/2013). J.C.M. Barreira and T. Visnevschi-Necrasov thank FCT, POPH-QREN and FSE for their grants (SFRH/BPD/72802/2010 and SFRH/BD/31734/2006, respectively).

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