

Advances in Isoflavone Profile Characterisation using Matrix Solid-phase Dispersion Coupled to HPLC/DAD in *Medicago* Species

Tatiana Visnevschi-Necrasov,^{a,b} João C. M. Barreira,^{b,c,*} Sara C. Cunha,^b Graça Pereira,^d Eugénia Nunes^a and M. Beatriz P. P. Oliveira^b

ABSTRACT:

Introduction – Analytical methods used in phytochemistry analysis are limited by the sample preparation step, which should ideally be fast, accurate, ecofriendly and achievable using low quantities of the sample. Matrix solid-phase dispersion (MSPD) may be a good alternative for combining extraction and purification procedures, thereby reducing the indicated limitations. **Objective** – Applying an MSPD extraction procedure coupled to high-performance liquid chromatography diode-array detection (HPLC/DAD) as an alternative methodology to evaluate isoflavone profiles.

Methods – Isoflavone profiles were determined for the leaves of nine species of *Medicago* in the late flower phenological stage (one or more nodes with 50% open flowers, no seed pods). Extraction was performed following MSPD, and isoflavone profiles were characterised using HPLC/DAD. The quantified amounts were compared with previous results in different species commonly recognised as good sources of isoflavones.

Results – Formononetin was the major isoflavone in most species, except *M. polymorpha* and *M. truncatula*. The isoflavone amounts were significantly different among the assayed species, with *M. orbicularis* and *M. arabica* as the major isoflavone sources, while *M. rigidula* presented the lowest contents. Furthermore, the detected differences allow electing the best species as a primary source of a specific isoflavone.

Conclusion – The MSPD allowed good extraction efficiency, reproducibility and recovery. Some of the species showed relevant isoflavone contents, even when compared with acknowledged plant sources such as soy or red clover. To the best of our knowledge the results presented are reported for the first time in these species. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: HPLC/DAD; MSPD; interspecific variation; isoflavones; *Medicago*

Introduction

The *Medicago* genus includes about 56 species, mainly distributed in moderate climate areas. The main species are widely present throughout the Mediterranean basin countries, including Portugal, Spain, France, Italy and Greece (Cocks, 2003; Farag *et al.*, 2007). These species are grown predominantly for phytoremediation and as sources of phytochemicals or high-quality forage for livestock (Seguin *et al.*, 2004). Their phytochemicals, including carotenoids, saponins or phytoestrogens, have been related with health benefits and disease prevention. Phytoestrogens are well-known examples of phytochemicals, belonging mainly to the large group of substituted phenolic compounds known as flavonoids (Yildiz, 2005). The biosynthesis of these compounds is dependent not only on the environment but also on genotypic factors (Hoeck *et al.*, 2000). The three main classes of phytoestrogens are isoflavones, lignans and coumestans, which are present in fruits, vegetables and whole grains. Isoflavones, the most extensively studied class of phytoestrogens, occur largely in soybeans (Jacobs *et al.*, 2009), mainly as genistin, daidzin and glycitin (Campos *et al.*, 2007). These compounds are documented as having a wide range of human health benefits, such as alleviation of menopausal symptoms (Jacobs *et al.*, 2009) and prevention of certain cancers (Jian, 2009), cardiovascular diseases (Wu and Muir, 2008) and osteoporosis (Sabudak and Guler, 2009).

Herbal preparations are known for having several bioactive compounds, and a high number of therapeutic agents derive directly (extraction and purification) or indirectly (chemical synthesis of naturally occurring substances) from plants and their preparations (Knoss and Chinou, 2012). The sample preparation procedure performed in the qualitative and quantitative analysis of herbal preparations is the limiting step in the whole analytical methodology. Ideally, sample preparation should be fast, accurate, environmentally friendly and achievable with low quantities of sample. Several techniques, such as percolation, Soxhlet extraction, ultrasound-assisted solvent extraction,

* Correspondence to: J. C. M. Barreira, Laboratório de Hidrologia e Bromatologia, Faculdade de Farmácia, Universidade do Porto, Portugal. E-mail: jbarreira@ipb.pt

^a CIBIO-ICETA, Faculdade de Ciências, Universidade do Porto, R. Padre Armando Quintas, 4485-661 Vairão, Portugal

^b REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, n° 228, 4050-313

^c CIMO-ESA, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal

^d INRB/IP - INIA - Instituto Nacional de Recursos Biológicos, Caia E São Pedro Estrada Gil Vaz, 7350-228 Elvas, Portugal

microwave-assisted solvent extraction, accelerated solvent extraction and supercritical fluid extraction, have already been used for isolation of phytochemicals (Klejduk *et al.*, 2004, 2010; Oniszczuk *et al.*, 2013). Matrix solid-phase dispersion (MSPD) may be a good alternative for combining the extraction and purification procedures (Visnevschi-Necrasov *et al.*, 2009), presenting also the soundest advantages of a quick, easy, cheap, effective, rugged and safe (QuEChERS) methodology (Bustamante-Rangel *et al.*, 2014). The MSPD procedure is used for the preparation, extraction and fractionation of compounds from solid, semi-solid, liquid and highly viscous biological samples. Furthermore, it allows performing homogenisation, extraction and clean-up stages simultaneously, eliminating most of the problems associated with the classic liquid–liquid and solid-phase extractions (Capriotti *et al.*, 2010). In addition, it has been used in the isolation of several analyte classes in different natural matrices (Barker, 2007; Wu *et al.*, 2009). Regarding isoflavones, MSPD has been successfully applied for their extraction in red clover (*Trifolium pratense*) (de Rijke *et al.*, 2004; Visnevschi-Necrasov *et al.*, 2009). After which, isoflavone profiling is usually based on high-performance liquid chromatography (HPLC) coupled with electrochemical, ultraviolet-visible (UV-Vis), diode-array (DAD), fluorimetric and/or mass spectrometric detection (Farag *et al.*, 2007). Although there are some reports, with emphasis in soybean, there are few published data and validated methods for isolating isoflavones in *Medicago* spp. In this work, a rapid, simple and inexpensive analytical tool was developed to evaluate isoflavone profiles in different *Medicago* species.

Experimental

Plant material

Nine species of *Medicago* (*M. arabica*, *M. doliata*, *M. minima*, *M. murex*, *M. orbicularis*, *M. polymorpha*, *M. rigidula*, *M. tornata* and *M. truncatula*) were sown in November 2008 at the Experimental Field of the University of Porto (Agrarian station of Vairão). The vegetal germplasm was obtained from the Portuguese collection of *Leguminosae* provided by the National Institute of Biological Resources (Instituto Nacional dos Recursos Biológicos, I.P.). Voucher specimens of each *Medicago* species were numbered and deposited in the local herbarium. Samples were collected from February to July in the late flower phenological stage (one or more nodes with 50% open flowers, no seed pods). For each species, three independent samples were selected (in different locations within the limits of the indicated Experimental Field) consisting in fresh leaves from randomly selected plants (five plants for each sample) belonging to two different accessions; samples were dried at 65°C during 72 h and milled, at a particle size of 0.1 mm, using an A11 analysis mill (IKA Werke, Staufen, Germany).

Chemicals and reagents

Acetonitrile (HPLC grade) and formic acid (analytical grade) were from Merck (Darmstadt, Germany). Purified demineralised water used was from a 'Seradest LFM 20' system (Seral, Ransbach-Baumbach, Germany). The eluents were filtered through 0.45 µm filters and degassed under reduced pressure and ultrasonic bath. Disposable syringe filter PTFE 0.45 µm was from Macherey-Nagel (Düren, Germany). The C₁₈-bonded silica (particle size 55–105 µm) used as sorbent for MSPD was from Waters (Milford, MA, USA).

Extraction procedure

The MSPD extraction of isoflavones was performed following a previous method with slight modifications (de Rijke *et al.*, 2004). An aliquot of

500 mg of the previously milled dried sample, 2 g of C₁₈ and 40 mg/kg of internal standard (IS, 200 µL at 100 mg/L) were placed in a glass mortar and blended with a glass pestle during 2–3 min. This mixture was then transferred to an empty column and connected to a vacuum system. The column was washed with 10 mL of distilled water (reddish-brown phase eluted from the column) and the isoflavones were eluted with 5 mL of methanol:water (9:1, v/v). Before HPLC analysis, the extracts collected in amber vials were filtered through a 0.45 µm polytetrafluoroethylene (PTFE) membrane (Visnevschi-Necrasov *et al.*, 2009). Different samples of two distinct accessions of all species were extracted.

HPLC determination of isoflavones

Purity-corrected individual isoflavones stock solutions (1 g/L) were prepared in methanol:water (75:25, v/v). A composite stock standard solution of multiple isoflavones containing 40 mg/L of each individual compound was prepared consisting of: biochanin A (≥97%), puerarin (≥99%), glycitein (≥97%), daidzein (≥98%), daidzin (≥95%), prunetin (≥98%), genistein (≥98%), genistin (≥95%) and formononetin (≥99%), obtained from Sigma-Aldrich (St Louis, MO, USA), and pratensein and irilone (both ≥98%) from Chromadex Inc. (Barcelona, Spain). The IS 2-methoxyflavone was obtained from Sigma. A working IS solution was prepared in methanol at 1 g/L. All the solutions were stored at -20°C in amber glass vials when not in use.

Chromatographic analyses were performed with a Jasco (Tokyo, Japan) high-performance liquid chromatograph equipped with a PU-2080 quaternary pump and a Jasco AS-950 automatic sampler with a 20-µL loop. Detection was performed with a Jasco model MD-2010 multi-wavelength diode-array detector (DAD). Chromatographic separation of the compounds was achieved with a Luna 5U C₁₈ column (5 µm, 150 × 4.60 mm; Teknokroma, Barcelona, Spain) operating at 40°C. The eluent was a gradient of acetonitrile (A) and 0.1% formic acid (B), at a flow rate of 1 mL/min, with a linear gradient as follows: 0 min 33% B, 7 min 45% B, 15 min 50% B, 25 min 60% B, 30 min 70% B, 35 min 0% B, 37 min 33% B, maintaining these conditions for 10 min and returning to the initial ones within 3 min. Data were analysed using the Borwin-PDA Controller Software (JMBS, France). Compounds were identified by chromatographic comparisons with authentic standards and UV spectra. Quantification was made by DAD at 254 nm based on the IS method.

The limits of detection (LOD) and quantification (LOQ) were calculated based on the minimum amount of target analyte that produced a peak with a signal-to-noise ratio of three and ten times the background chromatographic noise, respectively. For the determination of these parameters a mixture of standard solutions was used. The LODs and LOQs corresponding to the different isoflavones are shown on Table 1. These values ranging from 0.011 to 0.171 mg/L for LODs and from 0.037 to 0.569 mg/L for LOQs are in the lower limits of those reported in similar studies (Visnevschi-Necrasov *et al.*, 2009).

Statistical analysis

All extractions were performed in triplicate and each replicate was quantified twice. Data were expressed as mean ± standard deviation. The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA). The fulfillment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Kolmogorov–Smirnov with Lilliefors correction and the Levene's tests, respectively. In the cases where statistical significance differences were identified, the dependent variables were compared using Tukey's honestly significant difference (in homoscedastic samples) or Tamhane's T2 (in heteroscedastic samples) tests. Results obtained for puerarin were classified using a simple *t*-test for equality of means (after checking the equality of variances through a Levene's test), because there were fewer than three groups (puerarin was detected only in *M. arabica* and *M. tornata*). All statistical tests were performed at a 5% significance

Table 1. Calibration data, limits of detection (LOD) and quantification (LOQ) of the isoflavones analysed by HPLC/DAD at 254 nm

Isoflavones	t_R (min)	Calibration data		LOD (mg/L)	LOQ (mg/L)
		Equation	R^2		
Puerarin	5.1	$y = 0.2548x + 0.1759$	0.9995	0.070	0.235
Daidzin	6.8	$y = 1.259x + 2.045$	0.9968	0.171	0.569
Genistin	8.2	$y = 0.4892x + 0.3524$	0.9987	0.065	0.216
Daidzein	14.1	$y = 0.2754x + 0.1021$	0.9937	0.025	0.083
Glycitein	15.5	$y = 0.1158x + 0.07584$	0.9991	0.092	0.305
Genistein	19.2	$y = 1.257x + 1.254$	0.9954	0.028	0.095
Pratensein	20.4	$y = 0.4145x + 1.735$	0.9992	0.019	0.043
Formononetin	23.6	$y = 1.364x + 4.875$	0.9981	0.043	0.143
Irilone	24.5	$y = 0.1045x - 0.03519$	0.9973	0.041	0.137
Prunetin	28.7	$y = 0.4065x + 0.8871$	0.9955	0.039	0.130
Biochanin A	30.2	$y = 0.3068x + 0.1025$	0.9994	0.011	0.037

level using the SPSS software, Version 20.0 (IBM Corporation, Armonk, New York, USA).

Results and discussion

Method assessment

Linearity. Linearity and sensitivity of the HPLC analysis were determined and the method was validated by the instrumental repeatability, precision and accuracy, using a sample of *M. murex*. This species was selected due to its content in isoflavones, which correspond to the median value among the assayed plants. The linearity of the method was tested using seven levels (corresponding to 31.25–2000 µg/mL) of each standard solution. The quantity of added IS (2-methoxyflavone) was 50 µg, corresponding to 100 mg/kg in the sample for all samples and standards. The calibration curves were obtained by plotting the isoflavones/IS area ratio versus the concentration of isoflavones in the standard. The calculated correlation coefficients were higher than 0.99 (Table 1).

Recovery and repeatability. To assess the method's accuracy and repeatability, extractions were carried out at two fortification levels (50 and 100 mg/kg) and each test performed seven times. *Medicago murex* leaf samples were fortified with the target isoflavones in methanol:water (9:1, v/v) solution and left in contact for 30 min. Unspiked 'blank' samples were previously analysed to determine the presence of isoflavones. To calculate the recovery values, the contribution of each isoflavone in the 'blank' sample was subtracted from the corresponding value obtained in each spiked sample. In all fortification levels, recoveries were in the range of 82–104% (Table 2), whereas relative standard deviation (RSD) values ranged from 4% to 9%. The values obtained are similar to the recoveries reported by other authors using Solid-liquid extraction procedure for the analysis of isoflavones (Delmonte *et al.*, 2006; Saviranta *et al.*, 2008; Visnevschi-Necrasov *et al.*, 2009).

Isoflavone profiles

The first stage of the MSPD process, in which samples are structurally disrupted by manually blending with a solid-support phase (such as C_{18} sorbent, C_8 sorbent, synthetic polymers, Florisil), was effectively achieved by using the selected C_{18} sorbent.

Table 2. Average of recoveries and RSDs obtained for spiked samples analysed by HPLC/DAD at 254 nm ($n = 7$)

Isoflavones	Percentage recovery \pm %RSD	
	50 mg/kg	100 mg/kg
Puerarin	98 \pm 8	95 \pm 9
Daidzin	85 \pm 6	94 \pm 7
Genistin	82 \pm 5	99 \pm 4
Daidzein	92 \pm 4	97 \pm 6
Glycitein	88 \pm 7	92 \pm 4
Genistein	90 \pm 6	98 \pm 5
Pratensein	84 \pm 5	95 \pm 6
Formononetin	102 \pm 5	104 \pm 7
Irilone	95 \pm 7	100 \pm 8
Prunetin	87 \pm 6	92 \pm 5
Biochanin A	84 \pm 6	88 \pm 6

Accordingly, MSPD extraction was chosen because it allows performing the extraction and clean-up of extracts in a single step, requiring small amounts of sample (500 mg), low consumption of organic solvents (10 mL) and short extraction time (10 min), making it an advantageous alternative procedure for routine analysis (Visnevschi-Necrasov *et al.*, 2009), different to classic extraction methods that often require clean-up steps, large amounts of sample, sorbents and organic solvents, thus being expensive and time consuming (Barker, 2007; Capriotti *et al.*, 2010). Furthermore, MSPD does not require heating during the extraction, avoiding the possible degradation of thermolabile compounds and its chromatographic characteristics show that the elution of a single compound, several classes of compounds or complete fractionation of the sample matrix components can be performed (Oniszczuk *et al.*, 2013).

After this step, the selected solvent (methanol:water 9:1, v/v) allowed an appropriate elution of the column-packed mixture of sample and solid support. The possibility of analysing this eluent directly by HPLC simplifies the process and greatly reduces the time for sample preparation. The silica-based C_{18} -column used for chromatographic separation was chosen due to its good mechanical strength and reproducibility. In fact, the silica-based octadecyl material was previously reported as having higher reproducibility than C_8 -type sorbents (Oniszczuk *et al.*, 2013).

Following the comparative results previously obtained for isoflavone profiling in different species (Visnevski-Necrasov *et al.*, 2009), a gradient of acetonitrile (A) and 0.1% formic acid (B) was chosen for the elution, due to the solubility of isoflavones in this mixture and also because of the peak resolution obtained.

In terms of interspecific variability, the results obtained for the selected *Medicago* species (the accession numbers for each species are indicated in Table 3) revealed qualitative and quantitative differences in their isoflavone profiles (Table 3). An exemplifying HPLC phenolic profile, recorded at 254 nm, is presented in Fig. 1 for *M. murex*.

Most isoflavones were detected in their aglycone form (all chemical structures are shown in Fig. 2), which might be related to the early phenological stage in which the samples were obtained. Despite the close phylogenetic relationship among these plants, there is a great variability among the isoflavone profiles obtained. *Medicago orbicularis* and *M. arabica* produced the highest overall contents, while *M. rigidula* presented the lowest content. *Medicago tornata* showed interesting contents in puerarin (77 mg/kg DM), genistin (98 mg/kg DM) and biochanin A (36 mg/kg DM). Likewise, glycitein was more abundant in *M. murex* (75 mg/kg DM), while *M. polymorpha* proved to be the highest potential source of genistein (683 mg/kg DM), pratensein (47 mg/kg DM) and irilone (1704 mg/kg DM). Finally, prunetin showed maximum values in *M. arabica* (129 mg/kg DM), and formononetin (the most abundant individual isoflavone in all *Medicago* species except *M. polymorpha* and *M. truncatula*, in which irilone was highest) was especially abundant in *M. orbicularis* (2746 mg/kg DM). The number of quantified isoflavones also showed great heterogeneity, varying from three (in *M. polymorpha*) to ten (in *M. tornata*). Daidzin was detected only in *M. tornata* and puerarin in *M. arabica* and *M. tornata*, while genistein, formononetin and irilone were detected in all *Medicago* species (except formononetin in *M. polymorpha*).

Genistein is one of the most studied isoflavones, being especially abundant in soybean (Kumar *et al.*, 2009), and often reported as having anti-tumour effects (Fotsis *et al.*, 1997; Magee *et al.*, 2004). Furthermore, the detection of a high excretion of genistein in the urine of vegetarian subjects led to the suggestion that this isoflavone might contribute to the preventive action of a plant-based diet on chronic diseases (Fotsis *et al.*, 1997). Alongside this, irilone, is known for its resistance against almost all types of microbial degradation (Braune *et al.*, 2010), probably due to its methylenedioxy group attached to the aromatic A-ring (Fig. 2). Formononetin was also previously documented as influencing cell proliferation (Wang *et al.*, 1995).

Despite the observed differences among the different species, the amounts quantified in both accessions of each species were quite similar (Fig. 3), most likely because all samples were obtained in the same location (Experimental Field of the University of Porto of the Agrarian station in Vairão), preventing the effect of edaphoclimatic conditions.

As expected, the species studied proved to have higher isoflavone concentrations than non-legume plant species such as onion, carrot, white cabbage, cauliflower, lettuce, artichoke and grapefruit (Kuhnle *et al.*, 2007; Konar *et al.*, 2012). Regarding other *Leguminosae*, *M. orbicularis*, *M. arabica*, *M. polymorpha* and *M. murex* showed similar amounts when compared with focal isoflavone sources such as beans (Kuhnle *et al.*, 2007), soy or red clover (Klejdus *et al.*, 2007). In comparison with other *Medicago* species, the isoflavone profiles presented in this work proved

Table 3. Isoflavones contents (mg/kg dry matter) in the evaluated *Medicago* species. The results are presented as mean \pm SD

Species	Accessions identity	Puerarin	Daidzin	Genistin	Daidzein	Glycitein	Genistein	Pratensein	Formononetin	Irilone	Prunetin	Biochanin A
<i>Medicago arabica</i>	31, 32	3 \pm 1 ^b	ND	1.6 \pm 0.1 ^{ef}	9 \pm 1 ^c	ND	154 \pm 8 ^e	11.4 \pm 0.5 ^d	2606 \pm 92 ^b	77 \pm 3 ^{de}	129 \pm 6 ^a	ND
<i>Medicago dolata</i>	41, 42	ND	ND	6 \pm 1 ^c	11 \pm 1 ^a	ND	546 \pm 48 ^b	ND	1215 \pm 124 ^d	137 \pm 14 ^{cd}	ND	32 \pm 4 ^b
<i>Medicago minima</i>	67, 68	ND	ND	4 \pm 1 ^{cde}	ND	32 \pm 4 ^b	27 \pm 2 ^g	29 \pm 4 ^c	689 \pm 42 ^e	24 \pm 1 ^{ef}	53 \pm 6 ^b	ND
<i>Medicago murex</i>	62, 69	ND	ND	5 \pm 1 ^d	10 \pm 1 ^b	75 \pm 7 ^a	109 \pm 9 ^f	7.2 \pm 0.4 ^e	1526 \pm 70 ^c	75 \pm 5 ^{de}	5.7 \pm 0.5 ^d	16 \pm 2 ^c
<i>Medicago orbicularis</i>	66, 73	ND	ND	4.2 \pm 0.3 ^{cde}	ND	27 \pm 4 ^c	223 \pm 4 ^d	ND	2746 \pm 118 ^a	91 \pm 18 ^{cd}	ND	ND
<i>Medicago polymorpha</i>	82, 83	ND	ND	ND	ND	ND	683 \pm 46 ^a	47 \pm 5 ^a	ND	1704 \pm 118 ^a	ND	ND
<i>Medicago rigidula</i>	96, 97	ND	ND	17 \pm 1 ^b	ND	ND	34 \pm 3 ^g	ND	118 \pm 2 ^g	3.4 \pm 0.3 ^f	ND	33 \pm 2 ^{ab}
<i>Medicago tornata</i>	111, 116	77 \pm 9 ^a	3.4 \pm 0.4	98 \pm 6 ^a	ND	1.5 \pm 0.4 ^d	38 \pm 11 ^g	9 \pm 1 ^{de}	356 \pm 23 ^f	143 \pm 11 ^c	36 \pm 4 ^c	36 \pm 3 ^a
<i>Medicago truncatula</i>	123, 127	ND	ND	2.5 \pm 0.5 ^{def}	2.0 \pm 0.4 ^d	ND	392 \pm 27 ^c	38 \pm 3 ^b	619 \pm 86 ^e	831 \pm 78 ^b	ND	ND
Homocedasticity ¹ p-value		<0.001	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
One-way ANOVA ² p-value		<0.001	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

ND, not detected.

¹Homocedasticity among species was tested by means of the Levene test.

²p < 0.05 meaning that the mean value of the evaluated parameter of at least one species differs from the others (in this case multiple comparison tests were performed).

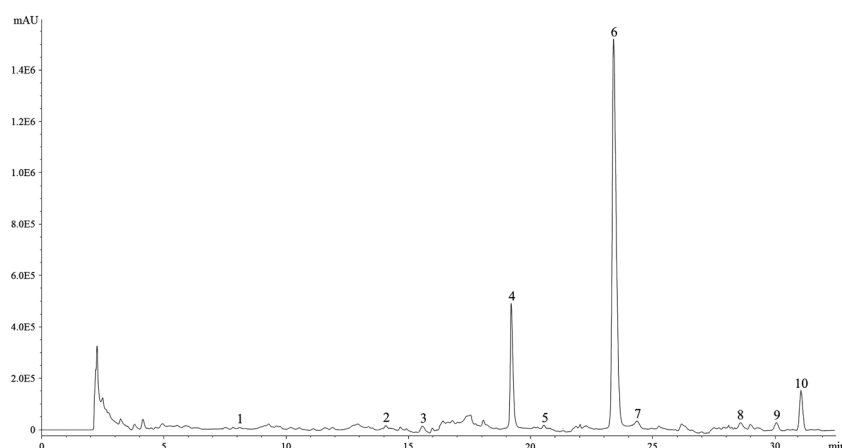


Figure 1. HPLC/DAD chromatogram of the isoflavones from *Medicago murex* recorded at 254 nm: 1, genistin; 2, daidzein; 3, glycitein; 4, genistein; 5, pratensein; 6, formononetin; 7, irilone; 8, prunetin; 9, biochanin A; 10, 2-methoxyflavone (IS).

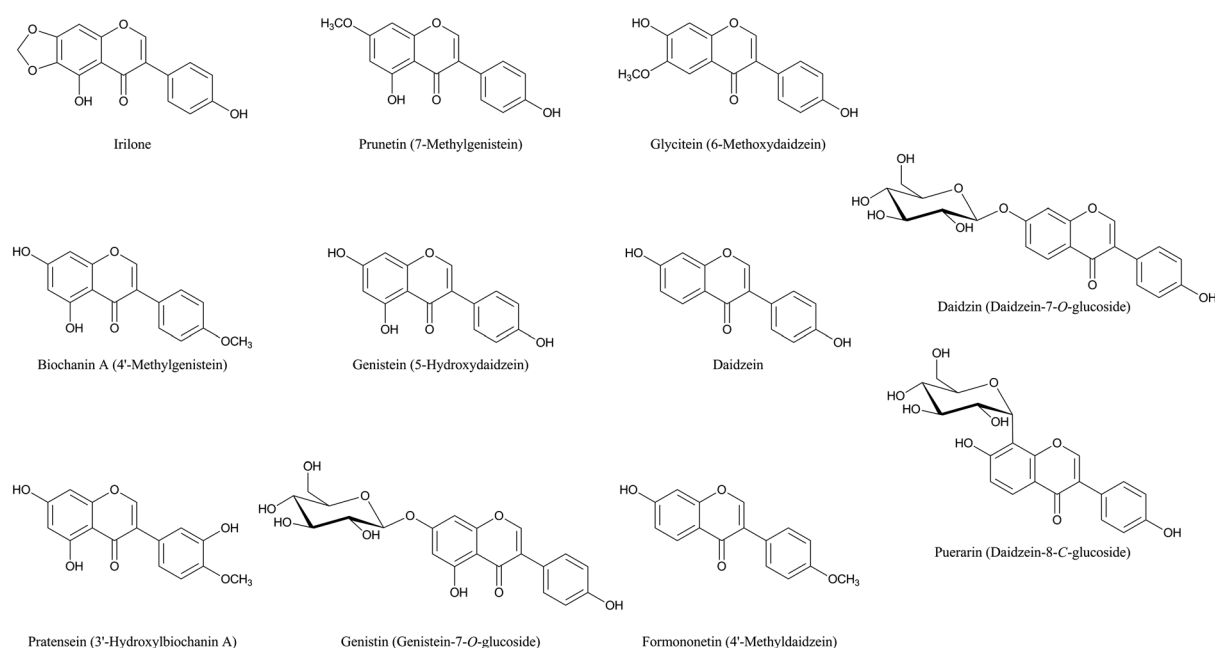


Figure 2. Chemical structures of the quantified isoflavones.

to be composed of a higher number of molecules (except *M. polymorpha* and *M. rigidula*) when compared with *M. sativa* sprouts (Silva *et al.*, 2013) and with *Medicago* spp. polar extracts (Rodrigues *et al.*, 2014).

In view of the high isoflavone contents presented by *Medicago* species, and particularly the amounts of genistein presented by *M. polymorpha*, *M. doliata* and *M. truncatula*, these plants might represent a new class of diet-derived anti-tumour compound sources. The newly found added value might act as an important enhancer to augment the production of this crop, granting a high potential natural resource. The different isoflavone profiles obtained for *Medicago* species might be important to define specific cultivation models for the production of determined isoflavones, considering the sustainability of their production and use, while valorising specific genotypes and

further enhancing the biological resources linked to the natural environment, improving the use of local plant material.

Overall, MSPD has demonstrated appropriate extraction efficiency, reproducibility and recovery, in comparison with conventional extraction techniques coupled with solid-phase extraction. Accordingly, it could be applied as an effective procedure for the determination of isoflavones in plant material. Knowing the isoflavone profiles in the *Medicago* species studied allows selecting the most suitable plant regarding the isolation of a specific isoflavone. This specificity is useful because different isoflavones have distinct effects on human health. Thereby, the applied extractive methodology was quantitatively advantageous and the detected profiles are valuable to select a determined *Medicago* species according to the potential health benefits of its isoflavones.

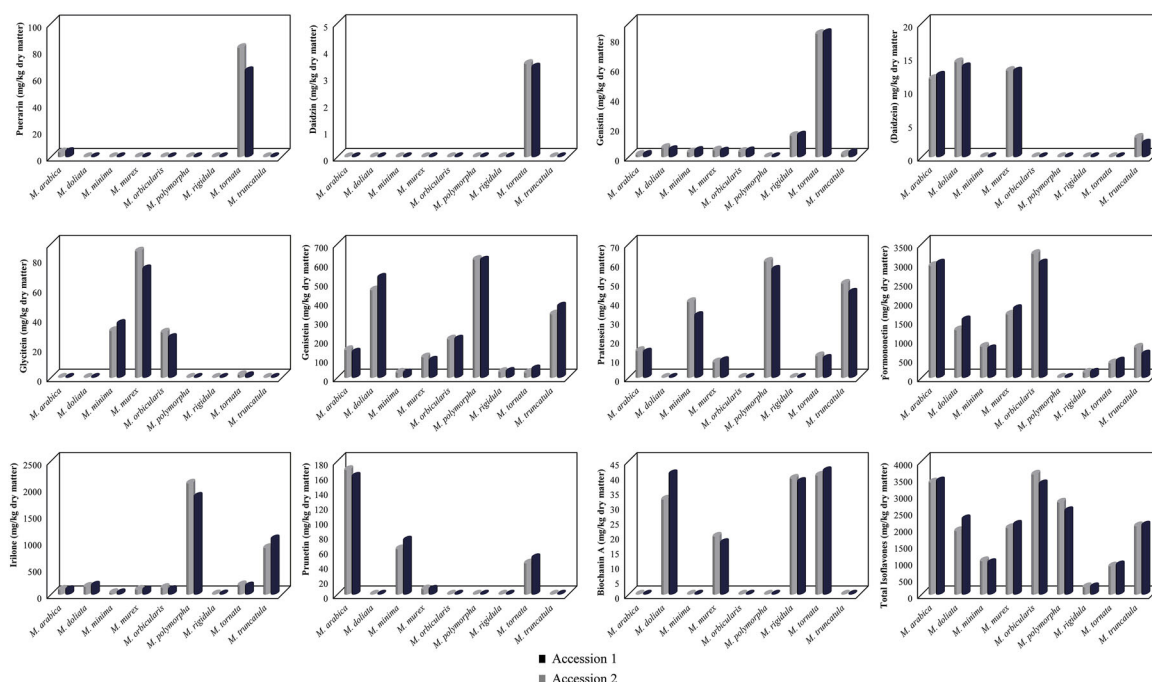


Figure 3. Individual isoflavone contents for each accession of the selected *Medicago* species.

Acknowledgements

T. Visnevschi-Necrasov and João C.M. Barreira are grateful to 'FCT - Fundação para a Ciência e a Tecnologia' for their grants (BD/31734/2006 and SFRH/BPD/72802/2010, respectively). S.C. Cunha is grateful to 'POPH-QREN - Tipologia 4.2, Fundo Social Europeu e Fundo Nacional MCTES'.

References

- Barker SA. 2007. Matrix solid phase dispersion (MSPD). *J Biochem Biophys Meth* **70**: 151–162.
- Braune A, Maul R, Schebb NH, Kulling SE, Blaut M. 2010. The red clover isoflavone irilone is largely resistant to degradation by the human gut microbiota. *Mol Nutr Food Res* **54**: 929–938.
- Bustamante-Rangel M, Pérez-Martín L, Delgado-Zamarreño MM. 2014. Comparative study of the methodology used in the extraction of isoflavones from legumes applying a modified QuEChERS approach. *Phytochem Anal* **25**: 170–177.
- Campos MG, Matos MP, Câmara MT, Cunha MM. 2007. The variability of isoflavones in soy seeds and the possibility of obtaining extracts for over the counter tablet preparations that can be standardized. *Ind Crop Prod* **26**: 85–92.
- Capriotti AL, Cavaliere C, Giansanti P, Gubbiotti R, Samperi R, Laganà A. 2010. Recent developments in matrix solid-phase dispersion extraction. *J Chromatogr A* **1217**: 2521–2532.
- Cocks PS. 2003. *The Adaptation of Perennial Legumes to Mediterranean Conditions: New Perennial Legumes for Sustainable Agriculture*. University of Western Australia Press: Crawley, WA.
- De Rijke E, de Kanter F, Ariesse F, Brinkman UAT, Gooijer C. 2004. Liquid chromatography coupled to nuclear magnetic resonance spectroscopy for the identification of isoflavone glucoside malonates in *T. pratense* L. leaves. *J Sep Sci* **27**: 1061–1070.
- Delmonte P, Perry J, Rader JI. 2006. Determination of isoflavones in dietary supplements containing soy, red clover and kudzu: extraction followed by basic or acid hydrolysis. *J Chromatogr A* **1107**: 59–66.
- Farag MA, Huffman D, Lei Z, Summer LW. 2007. Metabolic profiling and systematic identification of flavonoids and isoflavonoids in roots and cell cultures of *Medicago truncatula* using HPLC-UV-ESI-MS and GC-MS. *Phytochemistry* **68**: 342–354.
- Fotsis T, Pepper MS, Aktas E, Breit S, Rasku S, Adlercreutz H, Wahala K, Montesano R, Schweigerer L. 1997. Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. *Cancer Res* **57**: 2916–2921.
- Hoek J, Fehr W, Murphy P, Welke G. 2000. Influence of genotype and environment on isoflavone contents of soybean. *Crop Sci* **40**: 48–51.
- Jacobs A, Wegewitz U, Sommerfeld C, Grossklaus R, Lampen A. 2009. Efficacy of isoflavones in relieving vasomotor menopausal symptoms – a systematic review. *Mol Nutr Food Res* **53**: 1084–1097.
- Jian L. 2009. Soy, isoflavones, and prostate cancer. *Mol Nutr Food Res* **53**: 217–226.
- Klejdus B, Vacek J, Adam V, Zehnálek J, Kizek R, Trnková L, Kubáň V. 2004. Determination of isoflavones in soybean food and human urine using liquid chromatography with electrochemical detection. *J Chromatogr B* **806**: 101–111.
- Klejdus B, Vacek J, Benesova L, Kopecky J, Lapcik O, Kuban V. 2007. Rapid resolution HPLC with spectrometric detection for the determination and identification of isoflavones in soy proteins and plant extracts. *Anal Bioanal Chem* **389**: 2277–2285.
- Klejdus B, Lojková L, Plaza M, Šnobllová M, Štěrbová D. 2010. Hyphenated technique for the extraction and determination of isoflavones in algae: ultrasound-assisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry. *J Chromatogr A* **1217**: 7956–7965.
- Knoss W, Chinou I. 2012. Regulation of medicinal plants for public health – European Community monographs on herbal substances. *Planta Med* **78**: 1311–1316.
- Konar N, Poyrazoğlu ES, Köksal D, Artik N. 2012. Effect of different sample preparation methods on isoflavone, lignan, coumestan and flavonoid contents of various vegetables determined by triple quadrupole LC–MS/MS. *J Food Compos Anal* **26**: 26–35.
- Kuhnle GCC, Dell'Aquila C, Low YL, Kussmaul M, Bingham SA. 2007. Extraction and quantification of phytoestrogens in foods using automated solid-phase extraction and LC–MS/MS. *Anal Chem* **79**: 9234–9239.
- Kumar V, Rani A, Dixit AK, Bhatnagar D, Chauhan GS. 2009. Relative changes in tocopherols, isoflavones, total phenolic content, and anti-oxidative activity in soybean seeds at different reproductive stages. *J Agr Food Chem* **57**: 2705–2710.
- Magee PJ, McGlynn H, Rowland IR. 2004. Differential effects of isoflavones and lignans on invasiveness of MDA-MB-231 breast cancer cells *in vitro*. *Cancer Lett* **208**: 35–41.
- Oniszczuk A, Waksmundzka-Hajnos M, Skaliczka-Woźniak K, Głowniak K. 2013. Comparison of matrix-solid phase dispersion and liquid-solid extraction connected with solid-phase extraction in the quantification of selected furanocoumarins from fruits of *Heracleum leskowi* by high performance liquid chromatography. *Ind Crop Prod* **50**: 131–136.

- Rodrigues F, Almeida I, Sarmiento B, Amaral MH, Oliveira MBPP. 2014. Study of the isoflavone content of different extracts of *Medicago* spp. as potential active ingredient. *Ind Crop Prod* **57**: 110–115.
- Sabudak T, Guler N. 2009. *Trifolium* L. – review on its phytochemical and pharmacological profile. *Phytother Res* **23**: 439–446.
- Saviranta NMM, Anttonen MJ, von Wright A, Karjalainen RO. 2008. Red clover (*Trifolium pratense* L.) isoflavones: determination of concentrations by plant stage, flower colour, plant part and cultivar. *J Sci Food Agric* **88**: 125–132.
- Seguin P, Zheng W, Souleimanov A. 2004. Alfalfa phytoestrogen content: impact of plant maturity and herbage components. *J Agron Crop Sci* **190**: 211–217.
- Silva LR, Pereira MJ, Azevedo J, Gonçalves RF, Valentão P, de Pinho PG, Andrade PB. 2013. *Glycine max* (L.) Merr., *Vigna radiata* L. and *Medicago sativa* L. sprouts: a natural source of bioactive compounds. *Food Res Int* **50**: 167–175.
- Visnevschi-Necrasov T, Cunha SC, Nunes E, Oliveira MBPP. 2009. Optimization of matrix solid-phase dispersion extraction method for the analysis of isoflavones in *Trifolium pratense*. *J Chromatogr A* **1216**: 3720–3724.
- Wang W, Tanaka Y, Han Z, Higuchi CM. 1995. Proliferative response of mammary glandular tissue to formononetin. *Nutr Cancer* **23**: 131–140.
- Wu J, Muir AD. 2008. Isoflavone content and its potential contribution to the antihypertensive activity in soybean angiotensin I converting enzyme inhibitory peptides. *J Agr Food Chem* **56**: 9899–9904.
- Wu R, Han F, Shang J, Hu H, Han L. 2009. Analysis of patulin in apple products by liquid–liquid extraction, solid phase extraction and matrix solid-phase dispersion methods: a comparative study. *Eur Food Res Tech* **228**: 1009–1014.
- Yildiz F. 2005. *Phytoestrogens in Functional Foods*. Taylor & Francis: London; 210–211.