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ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE QUANTIFICATION OF SOME PHYTOESTROGENS IN PLANT MATERIAL

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Phytoestrogens are naturally occurring compounds found in plants to varying degrees. Epidemiological studies provided evidence for a protective role, especially of isoflavones, against the development of several chronic diseases, including cancers, cardiovascular diseases and osteoporosis. The highest amounts of isoflavones are found in soy, but they are also present in other beans and vegetables. A sensitive, high throughput UPLC-PDA method was elaborated for the simultaneous quantification of daidzein, genistein and coumestrol from plant materials. Sample preparation consisted in an ultrasound-assisted extraction, followed by acid hydrolysis and a final liquid-liquid extraction step in diethyl ether. Chromatographic separation was performed on a BEH C18 chromatographic column with a binary mixture of methanol/0.3% acetic acid as mobile phase, under gradient elution (flow rate = 0.7 mL/min). The total run time of analysis was 4 min. UV detection was performed at 244 nm for daidzein and coumestrol, and at 258 nm for genistein. The method was used to quantify selected phytoestrogens from several Leguminosae species, in order to identify other alternative phytoestrogen sources.

INTRODUCTION

Phytoestrogens are non-steroidal polyphenolic plant metabolites that, because of their structural similarities to 17β-estradiol, have the ability to bind to estrogen receptors and so they may exert estrogenic and/or anti-estrogenic effects. The major phytoestrogen groups are isoflavones, coumestans and lignans.[1] There is growing evidence that dietary phytoestrogens could have a role in prevention of estrogen-related cancers (breast cancer, prostate cancer), but also some beneficial effects regarding postmenopausal symptoms, osteoporosis and cardiovascular diseases.[1-12] However it has been reported in some scientific papers that phytoestrogens might have some detrimental effects on human health and there is need for further evaluation of their efficacy on cancer prevention.[13-17] Regarding their relative potencies (compared to estradiol), coumestrol (Figure 1) is the most potent phytoestrogen.[18,19] Isoflavones, are the most common form of phytoestrogens and, in plants, they are present mainly as inactive glycosides (β-glycosides, 6-O-acetyl-β-glycosides and 6-O-malonyl-β-glycosides) and only minimal amounts are present as the aglycone form. In the human body all glycosides are transformed into the pharmacologically active form, represented by the aglycones (daidzein, genistein and glycitein) (Figure 1).[12,20]

Isoflavones are widely distributed in the Fabaceae family. A great number of papers were published concerning the analysis of isoflavones from plant materials, especially soy (*Glycine max* L.), a very rich source of isoflavones (mainly daidzein and genistein).[20-30]

In order to isolate isoflavones from plant material or soy based foods, different extraction techniques were performed, from conventional ones (Soxhlet, shaking and stirring) to modern extraction methods, including supercritical fluid extraction, ultrasound-assisted extraction, pressurized liquid extraction (accelerated solvent extraction, pressurized solvent extraction), microwave-assisted extraction and solid phase extraction.[20-31]

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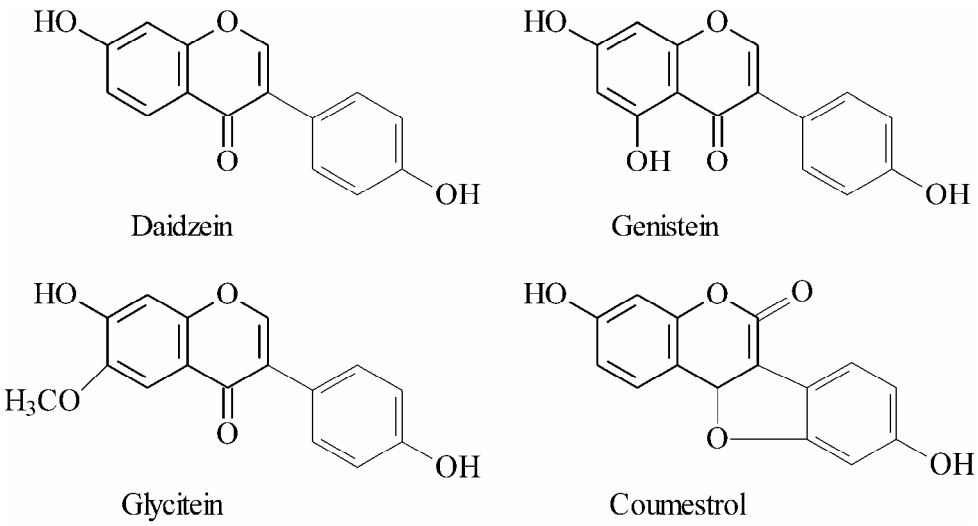

Fig. 1 – Chemical structure of the analyzed phytoestrogens.

The quantification of isoflavones can be performed with or without a hydrolysis step included. For methods without hydrolysis it is possible to quantify all glycosides and free aglycones present in the sample, but only if an adequate chromatographic separation is achieved. In this case the analysis can be more difficult because of the large number of analytes and also because of the stability issues of the glycosides during sample preparation. If the sample treatment involves hydrolysis (acid hydrolysis) all glycosides are transformed into free aglycones, and accurate quantification is performed more easily.

Several different analytical methods have been used for the analysis of isoflavones from plant materials and soy-based foods. These methods include gas chromatography, liquid chromatography, capillary electrophoresis and immunoassay. High-performance liquid chromatography (HPLC) with different detectors is the most common method used for the analysis of isoflavones. Given the advantages of columns with very small particle size of the packing material (1.7µm), [32,33] ultra-performance liquid chromatography was also used for isoflavone analysis. Several HPLC and UPLC assays for quantification of isoflavones in different *Leguminosae* species (especially in soy) are summarized in Table 1.

The main disadvantages of the HPLC methods are the very long chromatographic run time and the poor sensitivity. These drawbacks can be eliminated by using UPLC and chromatographic columns with particle size ≤ 1.8µm.[31,37]

The aim of our work was to elaborate a sensitive, high throughput UPLC-PDA method for the separation and for the simultaneous quantification of some isoflavone aglycones and coumestrol from different Leguminosae species, others than *Glycine max* L., in order to study these plants from the point of view of their isoflavone (and phytoestrogen) content. This type of study could be useful in order to identify other alternative isoflavone sources and to estimate their potential therapeutic utility/toxicity, based on the content in phytoestrogens.

RESULTS AND DISCUSSION

Regarding the analysis of isoflavones from plant materials, the analyst has two options: either to determine the total isoflavone content as free aglycones, after a previous hydrolysis step or to quantify separately each form of the isoflavones (aglycone, glycosides, and acetyl and malonyl esters). In this paper the analysis of some phytoestrogens from different plant species belonging to the *Leguminosae* (*Fabaceae*) family is performed after acid hydrolysis. Beside the reduction of the number of analytes, a hydrolysis step introduced before chromatographic analysis had also other reasons. Firstly, it eliminates the possible errors due to the instability of the glycoside forms (acetyl and malonyl derivatives are unstable during sample preparation). Secondly, it guarantees accurate quantification of the active forms of isoflavones (because only the aglycones are pharmacologically active and after ingestion, the conjugated forms are metabolized to these free aglycones). Finally, another issue with the quantification of glycosides is the availability of reference standards: due to their instability these forms are not widely commercially available.

Table 1

High-performance and ultra-performance liquid chromatography methods for determination of isoflavones in plant materials

Compounds	Sample	Column	Detection	LOD/LOQ	Chromatographic run time (min)	Ref.
Di, Gly, Gi, MGi	Soybeans	RP-18 LiChrospher 100, 5 µm	UV/ESI-MS	LOD = 1.3-2.1 mg/g	35 min.	21
De, Gle, Ge, Di, Gly, Gi, MDi, MGi, MGly	Soybeans	RP-18 LiChrospher 100, 250 mm, 4 µm	PDA	LOQ = 1177-1627 ng/mL	50 min.	23
De, Gle, Ge, Di, Gly, Gi, AcDi, AcGi, AcGly	Soy extracts	Two linked Chromolith Performance RP-18e, 100 mm x 4.6 mm	PDA	-	25 min	22
De, Gle, Ge, Di, Gly, Gi, AcDi, AcGi, AcGly, MDi, MGi, MGly	Soy extracts	Two linked Chromolith Performance RP-18e, 100 mm x 4.6 mm	PDA	LOQ = 1333-3140 ng/mL	25 min	25
De, Gle, Ge, Di, Gly, Gi, Ono, For, Sis, Bioch	Soy bits, roots, aerial parts, beans	Atlantis dC18, 20 mm x 2.1 mm, 3 µm	PDA	LOD = 1.1-9.4 ng/mL	8 min	26, 27
De Gle, Ge, Di, Gly, Gi, AcDi, AcGi, AcGly, MDi, MGi, MGly	Soybeans	Chromolith Performance RP-18e, 100 mm x 4.6 mm	PDA	LOQ = 1500-2100 ng/mL	15 min	29
De, Gle, Ge, Di, Gly, Gi, f For, Bioch, flavones, trihydroxyisoflavone, Cou	Soy and other *Leguminosae*	LC-18, 250 mm x 4.6 mm, 5 µm	MS/MS	LOD = 20 ng/g	35 min	34
Free and total De	*Genista* species	C18 Phenomenex, 50 mm x 4.6 mm, 3 µm	ESI-MS	LOD = 20 ng/mL	-	35
De Gle, Ge, Di, Gly, Gi, AcDi, AcGi, AcGly, MDi, MGi, MGly	Soy foods	Two linked Chromolith Performance RP-18e, 100 mm x 4.6 mm	PDA	LOQ = 800-1960 ng/mL	10 min	30
De, Gle, Ge, Di, Gly, Gi, Ono, For, Sis, Bioch	Soy preparations, *Trifolium pratense, Iresine herbstii, Ononis spinosa*	Zorbax SB C18, 30 mm x 2.1 mm, 1.8 µm	PDA	LOQ = 1.62-15.25 ng/mL	1.8 min	31
De, Ge	*Trifolium pratense*	Kromasil 250 mm x 4.6 mm, 5 µm	PDA	LOD=800ng/mL	30 min	36
De, Gle, Ge, Di, Gly, Gi, Ono, For, Sis, Bioch	*Trifolium pratense, Ononis spinosa, Pisum sativum, Glycine max*	Waters BEH C18 and BEH Phenyl, 50 mm x 2.1 mm, 1.7 µm; Zorbax SB-CN, 50 mm x 2.1 mm, 1.8 µm	PDA	LOD = 0.2-0.4 ng/mL	1.8-2.1 min	37
De, Ge, For, Bioch	*Trifolium species*	ODS Hypersil, 250 mm x 4.6 mm, 5µm	PDA	81-182 ng/mL	25 min	38
De, Ge, For, Bioch	*Trifolium species*	ODS Hypersil, 250 mm x 4.6 mm, 5 µm	PDA, FLD	103-357ng/mL (0.1ng/mL For - FLD)	75 min	39
De, Gle, Ge, Di, Gi, For, Bioch, puerarin, pratensein, pseudobaptigenin, irilone, prunetin	*Trifolium species*	Luna 5U C18, 150 mm x 4.6 mm, 5 µm	PDA	LOQ = 20-400 ng/mL	43 min	40

De – daidzein, Gle – glycitein, Ge – genistein, Di – Daidzin, Gly – glycitin, Gi – genistin, AcDi – acetyldaidzin, AcGly – acetylglycitin, AcGi – acetylgenistin, MDi – malonyldaidzin, MGly – malonylglycitin, MGi – malonylgenistin, For – formononetin, Bioch – biochanin A, Ono – ononin, Sis – sissotrin, Cou - coumestrol

In order to isolate the analytes, after hydrolysis, ultrasonic extraction with 50% ethanol for 20 min was performed. According to Rostagno *et al*[21], these conditions can guarantee the quantitative extraction of isoflavones.

Regarding the chromatographic analysis, the main technique used for isoflavone analysis is HPLC, using reversed-phase analytical columns. In an attempt to reduce the very long analysis time (see Table 1) several methods were reported in the literature, with the separation performed on monolithic columns. [22,25,29,30] With these columns higher flow rates can be used and so they permitted to reduce slightly the chromatographic analysis time. Even so, these methods are characterized by the consumption of large volumes of mobile phase.

A better alternative, in order to reduce analysis time, solvent consumption and also to increase sensitivity is to perform the analysis on a UPLC system, using a column filled with particles of 1.7 µm.

In this paper a UPLC method with PDA detection was elaborated for the quantification of some isoflavone aglycones and coumestrol from plant material. Regarding the UV detection, in order to select the optimum wavelengths for monitoring and to achieve maximum sensitivity, the UV spectra of all analytes were recorded in the range of 200-400 nm. Based on the spectral data (Figure 2) UV detection was chosen at 244 nm for daidzein and coumestrol, and at 258 nm for glycitein and genistein.

The chromatographic conditions described in the "Instrumentation and chromatographic conditions" section guaranteed the elution and good separation of daidzein, glycitein, genistein and coumestrol in less than 3 minutes. The retention times for the four analytes in these conditions were as follows: daidzein – 1.36 min, glycitein – 1.59 min, genistein – 1.90 min and coumestrol – 2.41 min (Figure 3). Due to some strongly retained compounds observed in case of plant extracts, it was necessary to include, after the elution of all analytes, a gradient with the decrease of aqueous component of the mobile phase to 0% (between 3 and 3.2 min).

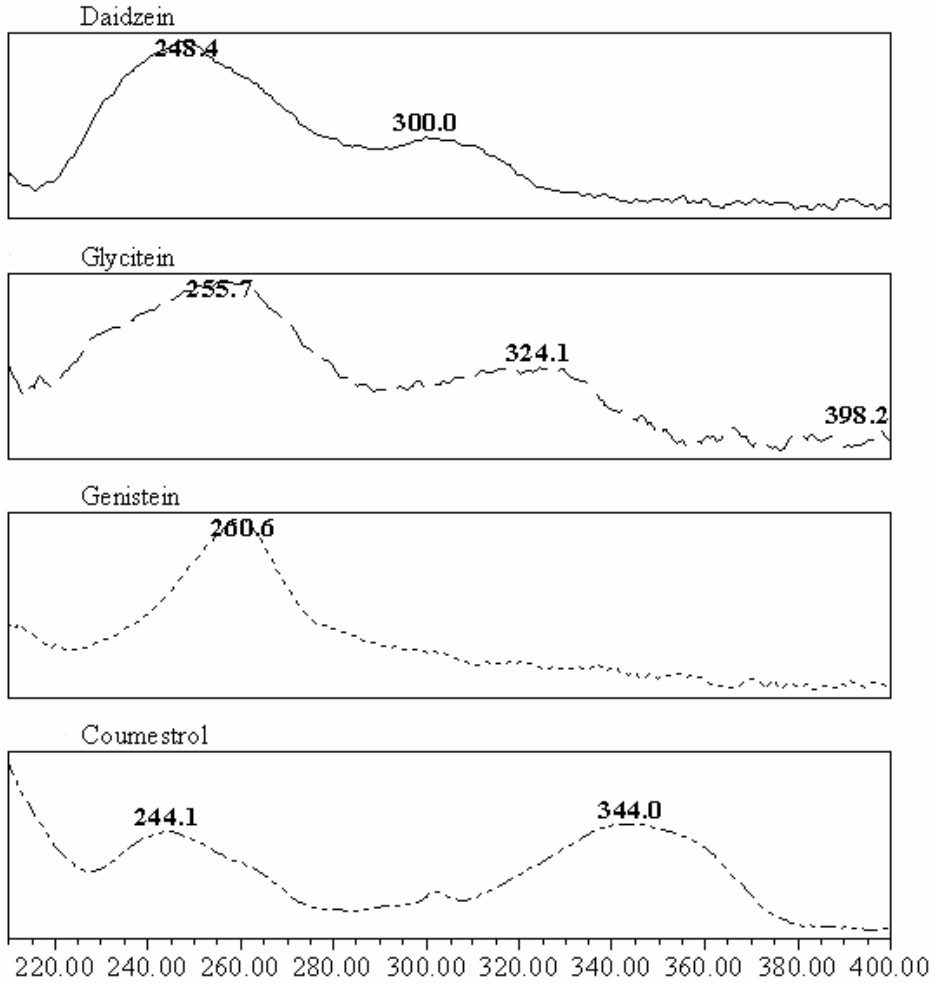

Fig. 2 – Ultraviolet spectra of the analyzed phytoestrogens.

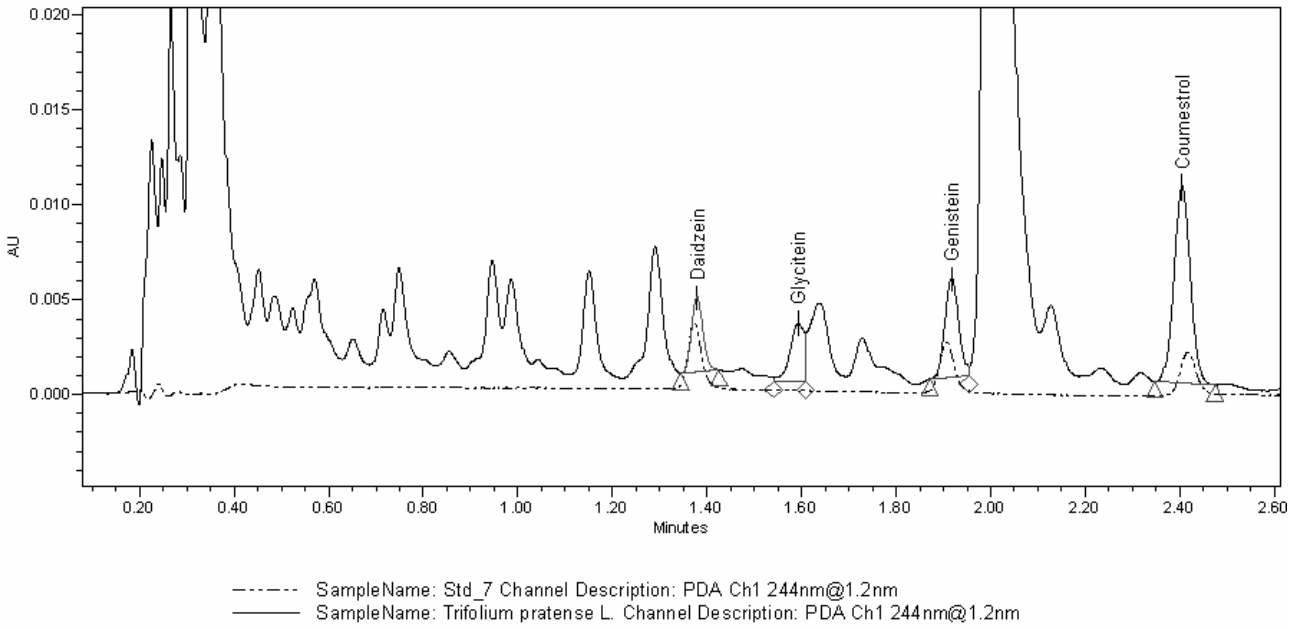

Fig. 3 – LC chromatograms of selected phytoestrogens from calibration standard solution (59.16 ng/mL De, 65.28 ng/mL Ge and 61.20 ng/mL Cou) and *Trifolium pratense* L. extract (λ = 244 nm).

This method was used to analyze hydrolyzed extracts obtained from aerial parts of *Trifolium pratense* L., *Trifolium repense* L., *Medicago lupulina* L., *Cytisus nigricans* L., *Genista sagittalis* L., *Genista tinctoria* L., *Cytisus albus* L., *Coronilla varia* L., *Lotus corniculatus* L. and *Dorycnium herbaceum* L. No interferences were observed in the chromatograms of these samples at the retention times corresponding to daidzein, genistein and coumestrol, but a significant interference existed in case of glycitein (Figure 3).

Due to this interference, in case of glycitein only qualitative analysis was possible (the confirmation of the presence of this aglycone in different plant materials could be possible based on retention time and UV spectra match).

In case of daidzein, genistein and coumestrol it was possible to use the elaborated method for quantitative purposes. The quantitative analysis of these three analytes, based on UV detection, was performed using an external standard method. Standard calibration in the 2 – 400 ng/mL (for daidzein and genistein) and 4-400 ng/mL (for coumestrol) range with good linearity ($r^2 > 0.999$) for a ten point plot was used to determine the concentration of phytoestrogens in plant samples. Calibration curves were constructed using a weighted (1/y) linear regression. Five calibration curves were constructed in five different days. The lower limit of quantitation was set at the level of the lowest calibrator in case of each analyte, based on the percent bias values obtained at this level. The LLOQ values were 1.97 ng/mL (percent bias % = -1.98–18.99), 2.18 ng/mL (percent bias % = -19.08–13.45) and 4.08 ng/mL (percent bias % = 5.74–19.83) for daidzein, genistein and coumestrol, respectively.

In order to identify possible new phytoestrogen sources a comparative study was performed on selected plant species from the *Fabaceae* family, based on their content in daidzein, genistein and coumestrol. The UPLC/UV analysis of the plant materials revealed varying concentrations for the selected compounds (Table 2).

The highest content of daidzein, genistein and coumestrol was found in *Genista tinctoria* L., indicating that this species could represent a valuable phytoestrogen source. Erdemoglu *et al*.[35] reported a total daidzein content in *Genista tinctoria* L. from the flora of Turkey of 7.00 μg/g. The aerial parts of *Genista tinctoria* L. collected from the wild flora of Romania showed significantly higher daidzein content (1632.00 μg/g). The second *Genista* specie studied presented also a significant content of the three selected phytoestrogens.

In case of *Trifolium pratense* L., the daidzein and genistein content were found to be a little bit lower than reported previously Zgorka *et al*[38,39], for the same species harvested in Poland. For *Trifolium repens* L. same authors reported[39] similar content of daidzein, but a lower content in genistein, than it was found in the same species analyzed in our study. Gatea *et al*. performed the quantification of daidzein and genistein in seeds,

inflorescence and buds of *Trifolium pratense* L. collected in Roumania. The daidzein content varied between 2.78 μg/g in the inflorescence and 467.24 μg/g in buds, while in case of genistein the results were between 2.46 μg/g (inflorescence) and 54.68 μg/g (mixture of bud and inflorescence).

Table 2

Concentration of selected phytoestrogens in the aerial parts of some Leguminosae (Fabaceae) species

Plant material	De(μg/g)	Ge (μg/g)	Cou (μg/g)
Trifolium pratense	52.70	126.00	273.60
Trifolium repense	16.80	50.30	11.20
Medicago lupulina	-	3.10	125.10
Cytisus nigricans	-	<LLOQ	66.00
Genista sagittalis	376.70	210.30	102.30
Genista tinctoria	1632.00	5290.30	464.00
Cytisus albus	24.10	112.10	<LLOQ
Coronilla varia	<LLOQ	21.40	<LLOQ
Lotus corniculatus	3.90	14.60	-
Dorycnium herbaceum	<LLOQ	9.80	-

In case of the other species included in this study, to our knowledge there are no data in the literature regarding their content in the selected phytoestrogens. The analysis of the phytoestrogen profile showed significant differences between these species. In case of *Medicago lupulina* L. and *Cytisus nigricans* L. coumestrol was observed as the major phytoestrogen, with very small content of genistein and lack of daidzein. *Cytisus albus* L., *Coronilla varia* L., *Lotus corniculatus* L. and *Dorycnium herbaceum* L. showed isoflavones as main phytoestrogens (especially genistein) and very low content or lack of coumestrol.

EXPERIMENTAL

Chemicals

Genistein and coumestrol standards were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), while daidzein standard from Fluka (Fluka Chemie GmbH, Buchs, Switzerland). Glycitein standard was purchased from ChromaDex (ChromaDex, Irvine, CA, USA). Acetonitrile and methanol (HPLC gradient grade) and acetic acid (analytical grade) were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Diethyl ether and hydrochloric acid (analytical grade) were obtained from Merck (Merck KgaA, Darmstadt, Germany). Distilled deionized water was produced by a Direct Q-3 Millipore (Millipore SA, Molsheim, France).

Standard solutions

Primary stock solutions of genistein, daidzein, coumestrol (0.2 mg/mL) and glycitein (0.05 mg/mL) were prepared in a mixture of double distilled deionized water/methanol (10/90, v/v) and stored at 4°C, protected from light. Intermediate working solutions were obtained through a ten-fold dilution of these primary stock solutions with a mixture of water/methanol (50/50, v/v). Calibration standard solutions were prepared daily by dilution of stock solutions with mobile phase.

Plant material

The plant material, consisting in aerial parts of *Trifolium pratense* L., *Trifolium repense* L., *Medicago lupulina* L., *Cytisus nigricans* L., *Genista sagittalis* L., *Genista tinctoria* L., *Cytisus albus* L., *Coronilla varia* L., *Lotus corniculatus* L. and *Dorycnium herbaceum* L. was collected from the wild flora of Roumania (Valea Draganului) in 2008, in the blooming period of the plants. Plant materials were dried at room temperature. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, "Iuliu Hatieganu" University of Medicine and Pharmacy.

Instrumentation and chromatographic conditions

The experiment was carried out using a Waters Acquity UPLC (Waters, Milford, MA, USA) system linked to a Acquity PDA (photodiode array)(Waters, Milford, MA, USA). The chromatographic separation was achieved on a Acquity BEH C18 column (Waters, Milford, MA, USA) (50 mm x 2.1 mm, 1.7 μm particle size), maintained at 60°C. The mobile phase consisted of a binary gradient prepared from a solution of 0.3% acetic acid (solvent A) and methanol (solvent B). The elution started with a linear gradient from 70% to 55% from start to 3 min, down to 0% A to 3.2 min and back to the initial conditions at 4 min, with re-equilibration during 1 min. All solvents were filtered through 0.5 μm filters (Millipore, SA, Molsheim, France) and degassed in an ultrasonic bath before use for UPLC. The mobile phase was delivered with a flow rate of 0.7 mL/min. The injection volume was set at 10 μL. The sample compartment was maintained at 4°C.

Chromatographic data acquisition and processing were performed using Empower 2 software (Waters, Milford, MA, USA).

Sample preparation

For the extraction of phytoestrogens from dry and powdered plant material we used the method developed by Rostagno *et al* (2003) with 50% ethanol using ultrasound-assisted extraction for 20 min. In these conditions they extracted quantitatively the isoflavones from soybeans.[21] A 5 g portion of dry and powdered plant material was subjected to this extraction procedure with 100 g of 50% ethanol. In order to release the aglycone form of the isoflavones, the extract was

treated with 2M hydrochloric acid (1/1, v/v) and maintained on a heated water bath at 70°C for 2h. After cooling, 2mL of the medium was extracted 2 times with 4 mL diethyl ether in a Multi Pulse vortexer (100 rpm) for 5 min and centrifuged at 3000 rpm for 6 minutes until the complete separation of the two phases. The organic phase was evaporated on a heated water bath (40°C) under nitrogen. The residue was dissolved in 5 mL of a mixture of water/methanol (50/50, v/v). This solution was diluted 10 fold with a mixture of 0.3% acetic acid/methanol (70/30, v/v) before the injection into the chromatographic column.

CONCLUSIONS

A UPLC-UV method was developed for the simultaneous quantification of daidzein, genistein and coumestrol from plant materials. Due to the short chromatographic run time and very good sensitivity this method proved to be adequate for high-throughput analysis of selected phytoestrogens. Using this assay the presence of daidzein, genistein and coumestrol was investigated in several plant species from the *Fabaceae* family. *Genista tinctoria* L. showed significantly higher content of selected phytoestrogens than other species included in this study, followed by *Genista sagittalis* L. and *Trifolium pratense* L. The present research, as part of a phytochemical study, provides precious information regarding the composition of the selected species and can contribute to the identification of some valuable phytoestrogen sources, as alternatives to soy.

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