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RESEARCH ARTICLE

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Quantitative determination of isoflavonoids in *Ononis* species by UPLC-UV-DAD

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Abstract

Introduction: The root of the *Ononis* species has been used internally and externally in ethnomedicine for centuries and contains biologically valuable isoflavonoid compounds. Therefore, it is important to obtain quantitative information about the isoflavonoid profile of these plants.

Objectives: In this article we aimed to develop an optimised sample preparation protocol alongside a validated method for the quantitative measurement of isoflavones, isoflavanones and pterocarpans in the form of glucosides and aglycones, in order to compare the specialised metabolites of *Ononis spinosa* L. and *O. arvensis* L.

Material and methods: Quantitative determination was carried out by the means of ultra-performance liquid chromatography coupled with ultraviolet diode-array detection (UPLC-UV-DAD).

Results: An optimised sample preparation method was developed to transform malonyl glucosides to their glucosidic forms. Chromatographic methods were created for the baseline separation of isoflavones, isoflavanones and pterocarpans alongside with their glucosides. Altogether 12 compounds were evaluated quantitatively in samples of *O. spinosa* and *O. arvensis*.

Conclusion: As a result, no characteristic change could be observed between the two species regarding their isoflavonoid pattern.

KEYWORDS

isoflavonoid, *Ononis*, quantitative, UPLC-UV-DAD

1 | INTRODUCTION

The most important structural classes of isoflavonoids are isoflavones, isoflavanones, pterocarpans, coumestans and rotenoids in diverse glycosidic forms.[1] The most detailed knowledge is available about isoflavones possessing phytoestrogenic properties, found in agriculturally important plants, such as soy, alfalfa, and red clover. However, there are many more plants in the *Leguminosae* family with different, but not less interesting isoflavonoid components. The subjects of our research are *Ononis* species, which grow around the Mediterranean region and have been used internally and externally in ethnomedicine for centuries.[2] Based on our previous work, both in *Ononis spinosa* L. and *O. arvensis* L., isoflavones, isoflavanones and pterocarpans are represented.[3–5] The roots of the plants were mainly used in traditional medicine as a diuretic agent, however, the isolated components proved other beneficial effects based on *in vitro* and *in vivo* test.[6,7] For example, formononetin showed neuroprotective effect,[8,9] pseudobaptigenin could activate peroxisome proliferator-activated

receptors,[10–13] sativanone could inhibit the enzymes α-glucosidase and hyaluronidase,[14,15] maackiain showed selective inhibition of monoamine oxidase-B[16] and medicarpin resulted to be effective against osteoporosis in animal models.[17–19] Regarding this information, we aimed to analyse the isoflavonoid profile of *Ononis* species quantitatively, in order to define their capacity to produce biologically valuable isoflavonoids.

Most research groups dealt with the quantitative characterisation of isoflavonoids in *Ononis* species, but the investigated compounds were limited to the ones with available commercial standards (namely genistein, biochanin A, daidzein and formononetin). These molecules are all representatives of the isoflavone group while isoflavanones and pterocarpans were not measured.[20–25] Furthermore, during our qualitative investigations,[3,4] evidence for the presence of genistein, daidzein and biochanin A could not be found. Thus, our aim was to develop an analytical method, which is suitable for the quantification of isoflavanones and pterocarpans beside isoflavones and to provide information about the levels of glucosides and aglycones, too.

Isoflavonoids are stored in plants in the forms of glucosides, glucoside malonates and aglycones,[1,26] so that the isoflavonoid spectrum is very heterogeneous. The simultaneous quantification of the plethora of metabolites from a single chromatographic analysis is almost impracticable, especially because of the lack of standard compounds. Another complicating factor is that the malonate esters are very unstable,[27] and their decomposition increases the level of glucosides. As a result, the most preferred approach is the quantification in the form of aglycones. However, the free aglycone content of the plant does not necessarily reflect the biological value, as the intestinal flora transforms the glucosides into aglycones.[28,29] Therefore, many research groups transform the glycosidic content into the corresponding aglycones.[23,25,29]

Herein we report an optimised sample preparation process and ultra-performance liquid chromatography coupled with ultraviolet diode-array detection (UPLC-UV-DAD) method for the quantitative determination of isoflavones, isoflavanones and pterocarpans in the form of glucosides and aglycones.

2 | EXPERIMENTAL

2.1 | Materials

Standard compound naringenin was purchased from Sigma-Aldrich (St Louis, MO, USA), formononetin, pseudobaptigenin, onogenin, sativanone, medicarpin and maackiain were purified from hydrolysed extracts of *O. spinosa* root in our laboratory. The isoflavone glucoside standards (formononetin-, pseudobaptigenin-, onogenin-, sativanone-, maackiain- and medicarpin-glucoside) were isolated in our laboratory, too. High-performance liquid chromatography (HPLC) and HPLC-mass spectrometry (MS)-grade methanol and acetonitrile were purchased from Fischer Scientific Co. (Fair Lawn, NJ, USA); LiChropure formic acid and acetic acid were obtained from Merck (Darmstadt, Germany). Purified water was prepared using a Millipore Direct-Q system (Millipore Corp., Bedford, MA, USA).

2.2 | Plant material

Ononis spinosa and *O. arvensis* samples were collected in their flowering periods from fields. *Ononis spinosa*: Dunaegyháza [(Hungary, 46°50′56.88″ N, 18°56′35.57″ E) Collected 1] and Hűvösvölgy [(Hungary, 47°33′22.88″ N, 18°58′33.89″ E) Collected 2)]. *Ononis arvensis* samples originated from Beregújfalu [(Ukraine, 48°17′21.1″ N, 22°48′08.7″ E) Collected 1)], Homoród-valley ((Romania, 46°10′29.1″ N, 25°25′22.5″ E; Collected 2)]. Commercial *O. spinosa* samples were obtained from Rózsahegyi Ltd (Erdőkertes, Hungary; Commercial 1), Biohorticulture Bio-Berta (Kiskőrös, Hungary; Commercial 2) and from Antica Erboristeria Romana Ltd (Rome, Italy; Commercial 3) and were received in a dried and chopped form according to the Ninth European Pharmacopoeia. Voucher specimens of collected samples were deposited in the Department of Pharmacognosy, Semmelweis University, Budapest, Hungary with voucher number 160725-On01, 150710-On03, 10720-OnArv01, 170727-OnArv02.

2.3 | Isolation of standard compounds

For the isolation of formononetin (3.5 mg, 97%), pseudobaptigenin (2.0 mg, 95%), onogenin (7.7 mg, 98%), sativanone (3.8 mg, 99%), medicarpin (5.1 mg, 98%) and maackiain (9.9 mg, 95%) the method described in our previous publication was used.[3–5] For the isolation of the glucosides, 50 g powdered wild-grown *O. spinosa* root sample was extracted with 200 mL 50% methanol, twice. The filtered liquid phases were unified and dried under reduced pressure. The residue was redissolved in 5 mL 30% methanol and purified using a CombiFlash NextGen 300+ (Teledyne ISCO, Lincoln, NE, USA) equipped with a RediSep Rf Gold C18 column (15.5 g). As eluent, methanol (solvent B) and 0.3% acetic acid (solvent A) were used with the following gradient programme: 0 min 30% B, 8 min 30% B, 10 min 60% B, 11 min 100% B and 18 min 100% B. The flow was set to 20 mL/min and 16 mL fractions were collected. The fractions eluting between 12 and 13 min were unified and further separated on a Hanbon Newstyle NP7000 preparative HPLC system with a Hanbon Newstyle NP3000 UV detector (Hanbon Sci. & Tech. Co., Jiangsu, China) equipped with a Gemini C18 reversed-phase column (150 mm × 21.2 mm i.d.; 5 μm, Phenomenex, Torrance, CA, USA) using eluents of 0.3% *v*/*v* acetic acid (A) and acetonitrile (B). Gradient elution was used with the following programme: 0 min 25% B, 15 min 25% B and 25 min 40% B with a 10 mL/min flow rate. The following peaks were collected: pseudobaptigenin 7-*O*-β-D-glucoside (13.3 min, 1.92 mg, 98%), formononetin 7-*O*-β-D-glucoside (14.1 min, 6.81 mg, 99%), onogenin 7-*O*-β-D-glucoside (17.6 min, 1.33 mg, 100%), sativanone 7-*O*-β-D-glucoside (19.7 min, 9.23 mg, 100%), maackiain 3-*O*-β-D-glucoside (20.6 min, 9.20 mg, 100%), medicarpin 3-*O*-β-D-glucoside (21.5 min, 4.07 mg, 98%). The identity of the compounds was verified by their retention time, ultraviolet (UV) detection, and tandem mass spectrometry (MS/MS) spectra. The purity of the samples was evaluated by HPLC.

2.4 | Preparation of stock solutions, calibration standards and quality control samples

Individual stock solutions of the standards were prepared dissolving the compounds in 70% methanol containing the internal standard (50 µL 2.0 mg/mL naringenin solution to 25 mL) to obtain ~1 mg/mL solutions. Equal parts of the standard solutions were mixed to gain the stock solution. Calibration standards were prepared by diluting the stock solution with the solution of the internal standard. The 10-point calibration curve was prepared using: 100 µg/mL, 60 µg/mL, 30 µg/mL, 10 µg/mL, 6 µg/mL, 3 µg/mL, 1 µg/mL, 0.6 µg/mL, 0.3 µg/mL, 0.1 µg/mL concentration levels. Quality control (QC) samples were prepared separately from the stock solution at 50 µg/mL, 5 µg/mL and 0.5 µg/mL nominal concentrations.

2.5 | Plant sample preparation

For quantitative analysis 0.100 g powdered plant material was weighted and 50 µL of the internal standard (2.0 mg/mL naringenin solution) was added first, then the samples were extracted with 5 mL 70% methanol by sonication for 30 min. The samples were centrifuged, and the pellet was repeatedly extracted twice more with the same method. The collected supernatants were filled up to 25 mL out of which 1 mL was filtered through a 0.22 µm polytetrafluoroethylene (PTFE) filter (Nantong FilterBio Membrane Co., Ltd, Nantong City, Jiangsu, China). From these 200 µL were taken out and kept at 83°C for 5 h prior to HPLC analysis. Optimising the extraction method, the amount of glucosides, glucoside malonates and aglycones were measured semi-quantitatively in three parallel samples. For testing the acidic hydrolysis 50 µL cc hydrochloric acid was added to the samples and the samples were kept at 83°C for 2 h. The samples treated with enzymatic hydrolysis were prepared from 0.10 g ground *O. spinosa* root mixed with 1.5 mL purified water or 1.5 mL of 1 mg/mL β-glucosidase solution and were let to sit for 24, 48 and 72 h. Thereafter, 3.5 mL methanol was added, and the same extraction method was used as mentioned earlier.

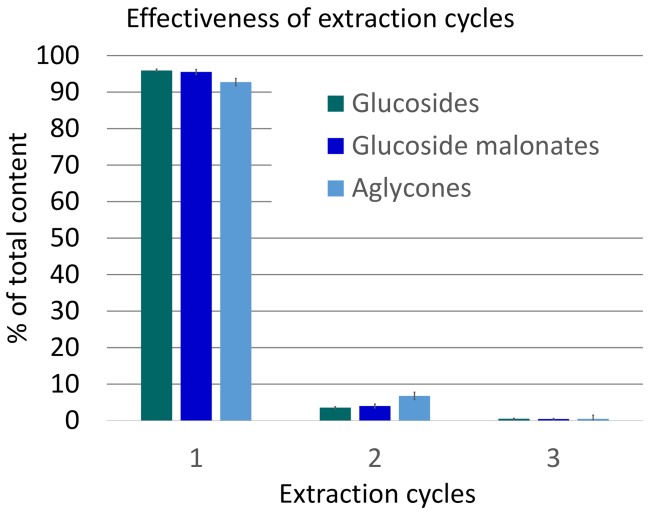

FIGURE 1 The relative quantity of isoflavonoid glucoside, glucoside malonates and aglycones through the first three extraction cycles [Colour figure can be viewed at wileyonlinelibrary.com]

2.6 | UHPLC-UV-DAD conditions for the quantitative analysis of *Ononis* samples

Quantitative measurements were executed on a Waters Acquity UPLC system (sample manager, binary solvent manager, PDA

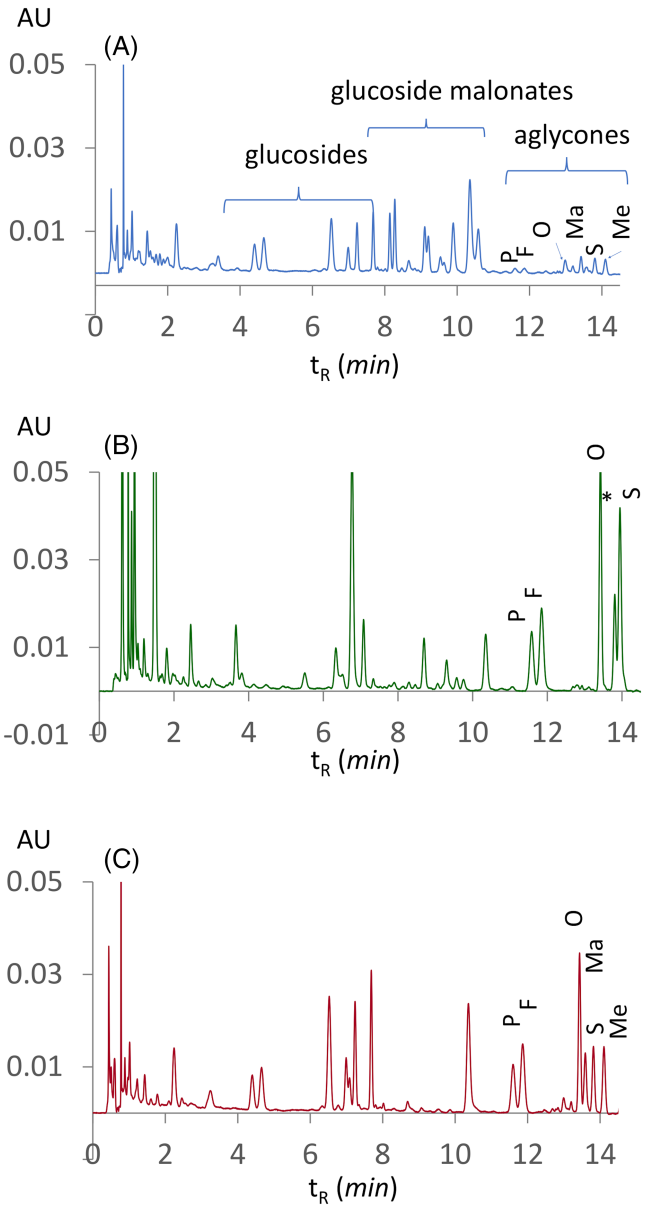

FIGURE 2 UPLC-UV-DAD chromatograms of *Ononis spinosa* extract recorded at 280 nm, showing the results of different hydrolytic approaches. (A) No treatment, (B) acidic hydrolysis, (C) enzymatic hydrolysis. F, formononetin; P, pseudobaptigenin; O, onogenin; Ma, maackiain; S, sativanone; Me, medicarpin; *, other compound [Colour figure can be viewed at wileyonlinelibrary.com]

detector) (Waters Corporation, Milford, MA, USA). The samples were analysed usinga Waters XSelect CSH Phenyl-Hexyl phase column (100 × 2.1 mm i.d; 3.5 µm; Waters Corporation, Milford, MA). For the semi-quantitative screening of the completeness of extraction cycles and different hydrolysis methods, the mobile phase consisted of 0.1% *v/v* formic acid (A) and acetonitrile (B). The gradient programme was as follows: 0 min 20% B, 5 min 20% B, 5.5 min 28% B, 11.5 min 28% B, 12 min 35% B, 20 min 35% B with 0.4 mL/min flow rate and 5 µL injected volume, the column temperature was set to 27°C. Aiming the determination of isoflavone derivatives, the same eluents were used, with the following gradient programme: 0 min 10% B, 15 min 30% B, 17 min 100% B, 19 min 10% B with 0.4 mL/min flow rate and 5 µL injected volume, the column was heated to 40°C. Quantifying the isoflavanone and pterocarpan derivatives, the following gradient was used: 0 min 25% B, 5 min 25% B, 6 min 29% B, 15 min 29% B, 17 min 100% B, 19 min 25% B with 0.4 mL/min flow rate and 5 µL injected volume, the column was heated to 27°C.

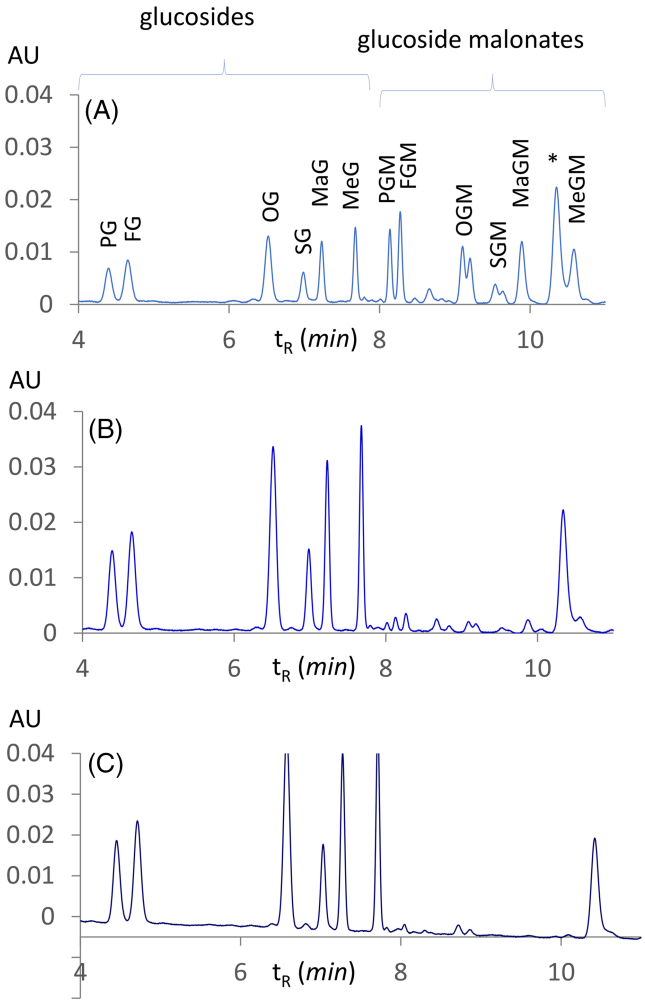

FIGURE 3 UPLC-UV-DAD chromatograms of *Ononis spinosa* extract recorded at 280 nm, showing the effect of heating time to the level of glucosides, and glucoside malonates. (A) No treatment, (B) 2 h 83°C, (C) 5 h 83°C. PG, pseudobaptigenin 7-*O*-glucoside; FG, formononetin 7-*O*-glucoside; OG, onogenin 7-*O*-glucoside; SG, sativanone 7-*O*-glucoside; MaG, maackiain 3-*O*-glucoside; MeG, medicarpin 3-*O*-glucoside; PGM, pseudobaptigenin 7-*O*-glucoside 6″-*O*-malonate; FGM, formononetin 7-*O*-glucoside 6″-*O*-malonate; OGM, onogenin 7-*O*-glucoside 6″-*O*-malonate; SGM, sativanone 7-*O*-glucoside 6″-*O*-malonate; MaGM, maackiain 3-*O*-glucoside 6″-*O*-malonate; MeGM, medicarpin 3-*O*-glucoside 6″-*O*-malonate [Colour figure can be viewed at wileyonlinelibrary.com]

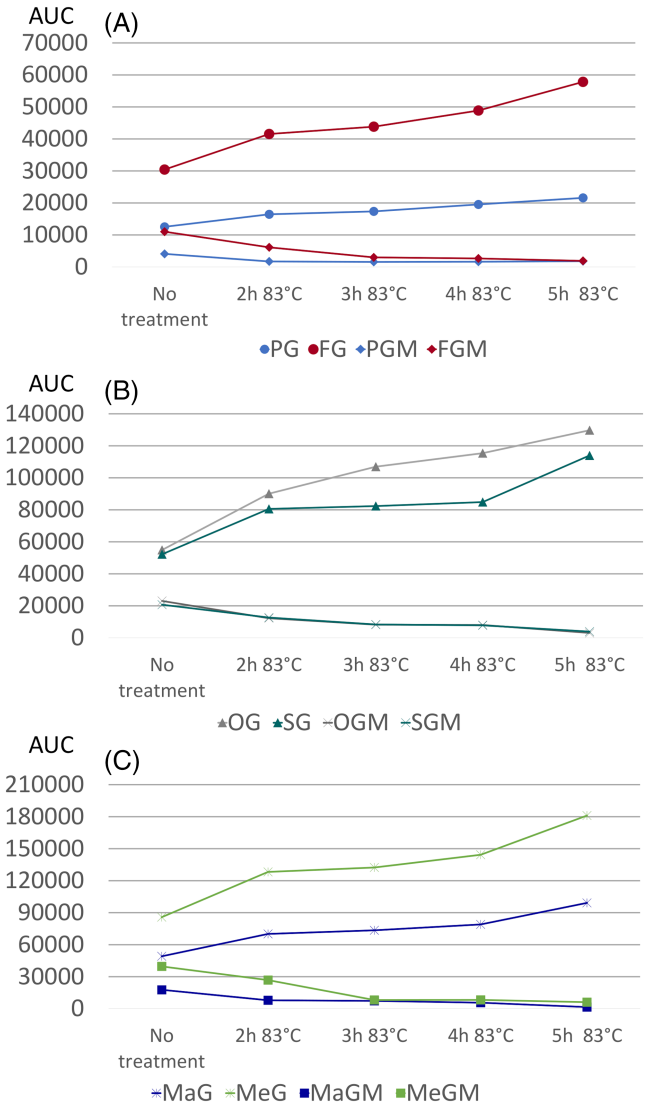

FIGURE 4 The change of the level of isoflavone (A), isoflavanone (B) and pterocarpan (C) glucosides and glucoside malonates depending on hydrolysis time. PG, pseudobaptigenin 7-*O*-glucoside; FG, formononetin 7-*O*-glucoside; OG, onogenin 7-*O*-glucoside; SG, sativanone 7-*O*-glucoside; MaG, maackiain 3-*O*-glucoside; MeG, medicarpin 3-*O*-glucoside; PGM, pseudobaptigenin 7-*O*-glucoside 6″-*O*-malonate; FGM, formononetin 7-*O*-glucoside 6″-*O*-malonate; OGM, onogenin 7-*O*-glucoside 6″-*O*-malonate; SGM, sativanone 7-*O*-glucoside 6″-*O*-malonate; MaGM, maackiain 3-*O*-glucoside 6″-*O*-malonate; MeGM, medicarpin 3-*O*-glucoside 6″-*O*-malonate [Colour figure can be viewed at wileyonlinelibrary.com]

3 | RESULTS AND DISCUSSION

3.1 | Quantification of isoflavonoids in *O. spinosa* and *O. arvensis* samples and *in vitro* cultures

3.1.1 | Sample preparation

Isoflavonoids are stored in plants mainly in their glycosidic forms (as glucosides or glucoside malonates),[1] however, only the aglycones can serve as phytoalexins, which can be found in the plant samples usually in lower amounts.[3] To start with, the effectiveness and completeness of the extraction was verified. As can be seen in Figure 1, following the third extraction cycle with 70% methanol, the quantity of residual isoflavonoids was negligible. Since many research groups choose acid hydrolysis in order to cleave the glycosidic bond and transform all derivatives to aglycones,[23,25,29] in a series of experiments, we aimed to determine the optimal concentration of hydrochloric acid and hydrolysis time. Unfortunately, the hydrolysis of pterocarpans was not successful. The amount of pterocarpan aglycones did not increase with time as expected, but the level of aglycones decreased.

As can be seen in Figure 2(A, B), there are still remnant isoflavone glucosides, whereas the pterocarpan aglycones decompose after 2 h of heating at 83°C with hydrochloric acid. A further approach for the hydrolysis of the glucosides is to use β-glucosidase enzymes,[30] either from an external source or based on the plant's endogenous β-glucosidase.[31] Treating the samples with water to activate the endogenous glucosidase enzymes could free the aglycones from their glycosidic storage, but this reaction was not quantitative. As can be seen in Figure 2(C), the malonates were erased from the sample and the level of the aglycones rose, while the amount of the glucosides dropped, but still a reasonable quantity of glucosides remained in the samples. The completeness of hydrolyses could not be reached with a longer time span (24, 48 or 72 h), nor heating the sample (25 or 37°C). Attempts were made to add an external enzyme (β-glucosidase isolated from almonds), but the total cleavage of the glucosides was not achieved. Interestingly, the hydrolysis of isoflavone (formononetin, pseudobaptigenin) glucosides was always of higher degree than isoflavanones and the least prone to hydrolyses were pterocarpan (medicarpin, maackiain) glucosides. As the transformation of all derivatives to the form of aglycones could not be realised, we aimed to simplify the samples to the stable forms of isoflavonoids: glucosides and aglycones. Rijke *et al.*[27] published, that isoflavonoid glucoside malonates can be selectively hydrolysed to glucosides with heating the sample to 83°C for 2 h. In our case, the malonates did not degrade completely under 2 h, but with the elongation of the heating time to 5 h, the level of malonates could be suppressed under the limit of quantitation (LOQ) (Figure 3). Under the 5-h time span, the glucosides did not show any degradation (see Figure 4).

3.1.2 | Optimisation of the chromatographic method

During the optimisation of the chromatographic method, the baseline separation of 12 compounds was aimed. These compounds could be grouped as glucosides and aglycones or isoflavones, isoflavanones and pterocarpans. Unfortunately, the perfect separation of all these compounds could not be realised. Various stationary phases (C18, phenyl-hexyl), eluent systems (methanol, acetonitrile), eluent flows (0.3–0.5 mL/min), gradient programmes and temperatures were tested, but our results showed that the modifications which ameliorated the separation of isoflavones (both glucosides and aglycones)

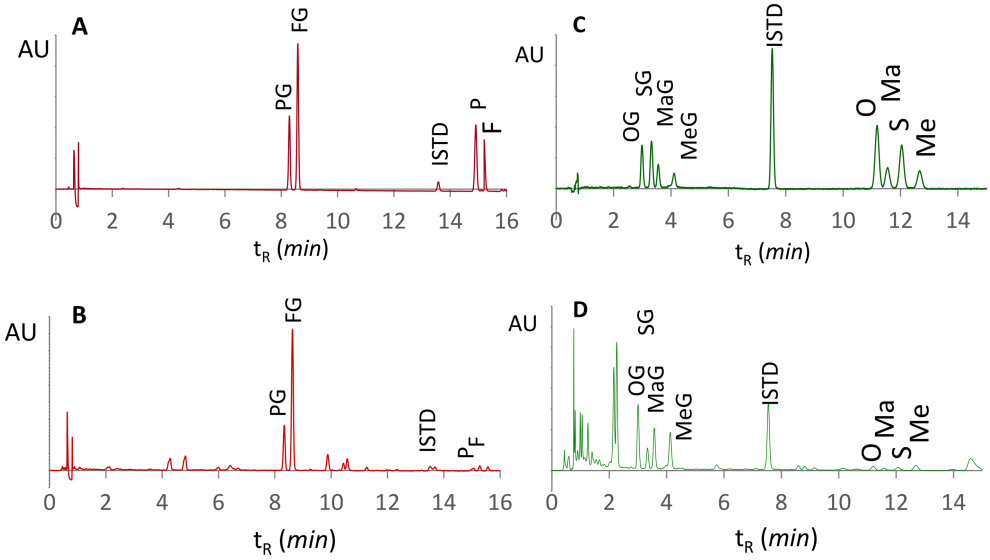

FIGURE 5 Chromatograms optimised for the separation of isoflavone derivatives [(A) standard compounds, (B) *Ononis spinosa* extract; both recorded at 249 nm] and for the separation of isoflavanones and pterocarpans [(C) standard compounds, (D) *O. spinosa* extract; both recorded at 288 nm]. PG, pseudobaptigenin 7-*O*-glucoside; FG, formononetin 7-*O*-glucoside; OG, onogenin 7-*O*-glucoside; SG, sativanone 7-*O*-glucoside; MaG, maackiain 3-*O*-glucoside; MeG, 3-*O*-glucoside; F, formononetin; P, pseudobaptigenin; O, onogenin; Ma, maackiain; S, sativanone; Me, medicarpin [Colour figure can be viewed at wileyonlinelibrary.com]

caused the overlapping of pterocarpans and isoflavanones. While the separation of isoflavones required higher temperature and lower organic modifier ratio, the decrease of acetonitrile concentration under 25% circumvented the separation of onogenin and maackiain. Thus, two methods were optimised based on the preliminary assays, one for the determination of isoflavone derivatives, and one for isoflavanones and pterocarpans, which enabled the baseline separation of compounds within 16 min. Peak assignments were made with single compound injections and UV spectral data. The following figure (Figure 5) shows the separation of the standard compounds of different skeletons and the sample extracts.

3.1.3 | Validation of the method

The calibration was based on the triplicate analysis of each working solution at 10 concentration levels. All peaks were integrated at their absorption maxima. The calibration curves were plotted using a 1/*x*-weighted linear model for the regression of peak area vs. analyte concentration. The determined linearity ranges can be seen in Table 1 along with the regression equations and the coefficients of determination. The limit of detection (LOD) and LOQ values were determined at 3 and 10 times the signal-to-noise ratio, respectively (Table 1). The concentrations of high, medium, and low QC samples for each standard can be seen in Table 1 with the intra- and inter-assay accuracy (deviation from nominal concentration) and precision [relative standard deviation (RSD)] values (n = 3). All results fulfilled the requirements of the FDA and EMEA guidelines of bioanalytical method validation, as the accuracy and precision values did not exceed ±15%, or ±20% in the case of low QC samples at the LOQ.

3.1.4 | Isoflavonoid levels in *O. spinosa* and *O. arvensis*

Regarding the sum of different isoflavonoids, the overall content ranged from 1.76 to 3.63 g/100 g with a mean of 2.31 g/100 g in the wild-grown root samples (Table 2). All samples contained a significantly higher amount in glycosidic form of the six isoflavonoids (Student's t-test, α = 0.05) (Figure 6). In average, the glycosides could be found at a 13.4 times higher level, than the aglycones, ranging from 1.4 to 37.1 times higher levels. One commercial *O. spinosa* sample

TABLE 1 Calibration curves of standard compounds

Name of compound	Linearity range (μg/mL) (number of points)	Equation	R^2	LOD (μg/mL)	LOQ (μg/mL)
Pseudobaptigenin 7-*O*-β-D-glucoside	0.1–100 (10)	$y = 0.1341x - 0.00003$	0.9994	0.060	0.200
Formononetin 7-*O*-β-D-glucoside	0.1–100 (10)	$y = 0.2811x - 0.00017$	0.9989	0.027	0.093
Onogenin 7-*O*-β-D-glucoside	0.3–100 (9)	$y = 0.1022x - 0.00158$	0.9989	0.042	0.141
Sativanone 7-*O*-β-D-glucoside	0.3–100 (9)	$y = 0.1921x - 0.00041$	0.9987	0.083	0.277
Maackiain 3-*O*-β-D-glucoside	0.3–100 (9)	$y = 0.0618x + 0.00189$	0.9991	0.058	0.193
Medicarpin 3-*O*-β-D-glucoside	0.6–100 (8)	$y = 0.0331x - 0.00247$	0.9973	0.165	0.552
Pseudobaptigenin	0.1–100 (10)	$y = 0.2337x - 0.00055$	0.9936	0.041	0.135
Formononetin	0.1–100 (10)	$y = 0.2026x - 0.00178$	0.9992	0.045	0.150
Onogenin	0.3–100 (9)	$y = 0.1608x + 0.00276$	0.9995	0.041	0.137
Sativanone	0.3–100 (9)	$y = 0.1852x - 0.00402$	0.9994	0.039	0.131
Maackiain	0.3–100 (9)	$y = 0.0765x + 0.00301$	0.9986	0.087	0.289
Medicarpin	0.6–100 (8)	$y = 0.0641x - 0.00408$	0.9995	0.123	0.406

LOD, limit of detection; LOQ, limit of quantitation.

TABLE 2 Mean isoflavonoid content in *m/m %* of *Ononis spinosa* and *O. arvensis* root extracts (n = 3)

	PG	FG	OG	SG	Ma	Me	P	F	O	S	Ma	Me	Total
O. spinosa													
Collected 1	0.171	0.374	0.250	0.093	0.503	0.706	0.014	0.048	0.016	0.016	0.020	0.038	2.25
Collected 2	0.235	0.342	0.281	0.086	0.402	0.492	0.017	0.023	0.014	0.014	0.022	0.035	1.96
Commercial 1	0.050	0.106	0.351	0.142	0.498	0.938	0.004	0.013	0.021	0.014	0.024	0.057	2.22
Commercial 2	0.293	0.429	0.597	0.166	0.758	1.128	0.015	0.053	0.050	0.027	0.044	0.066	3.63
Commercial 3	0.053	0.094	0.275	0.114	0.416	0.782	0.005	0.015	0.022	0.016	0.030	0.046	1.87
O. arvensis													
Collected 1	0.225	0.270	0.532	0.118	0.574	0.608	0.006	0.017	0.022	0.018	0.015	0.048	2.45
Collected 2	0.253	0.421	0.299	0.067	0.254	0.327	0.011	0.049	0.016	0.011	0.022	0.027	1.76

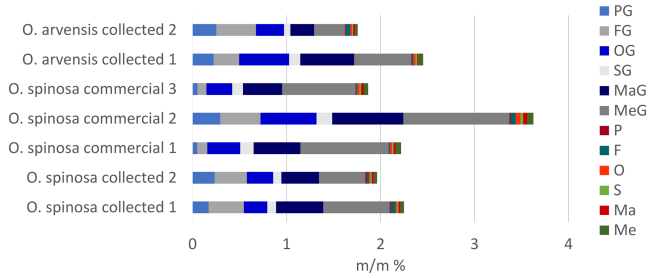

FIGURE 6 Absolute quantity of isoflavonoids in *Ononis spinosa* and *O. arvensis* samples [Colour figure can be viewed at wileyonlinelibrary.com]

showed a significantly higher total isoflavonoid content (Commercial 2, Figure 6), than all the other samples [one-way analysis of variance (ANOVA), Bonferroni *post hoc* test]. However, investigating the samples altogether no significant difference could be found between the two species regarding their total isoflavonoid content (Students *t*-test, $\alpha = 0.05$).

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