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RESEARCH ARTICLE

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Phytochemical analysis of *Ononis arvensis* L. by liquid chromatography coupled with mass spectrometry

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Abstract

Ononis arvensis L. can be found overall in Europe and is used to treat infections of the urinary tract and skin diseases in ethnopharmacology. Flavonoids, hydroxycinnamic acids, oxycoumarin, scopoletin and scopolin, phytosterols, lectins, and some selected isoflavonoids were identified in *O. arvensis* till date; however, there is a lack of the detailed investigation of the isoflavonoid profile of the plant. With the application of high-resolution tandem mass spectrometry, the fragmentation patterns of isoflavonoid derivatives found in *O. arvensis* roots and aerial parts were investigated and discussed. Isoflavonoid glucosides, glucoside malonates, aglycones, and beta amino acid derivatives were characterized, among which homoproline isoflavonoid glucoside esters were described for the first time. Besides the known isoflavonoid aglycones described earlier in other *Ononis* species, two 2′-methoxy isoflavonoid derivatives were detected. The presence of licoagroside B was verified, and its structure was also corroborated by NMR experiments. Altogether, the high-resolution fragmentation pattern of 47 isoflavonoids and glycosides is presented, and their relative quantity in the roots and the aerial parts can be evaluated. Based on this information, the chemotaxonomic relation of *Ononis* species and the biosynthesis of their compounds could be comprehended to a greater depth.

KEYWORDS

fragmentation, HPLC-ESI-MS/MS, isoflavonoid, *Ononis*, UHPLC-ESI-Orbitrap-MS/MS

1 | INTRODUCTION

The members of the *Ononis* genus, which belongs to the family Leguminosae, are natively distributed in Europe, Central Asia, and North Africa. *Ononis arvensis* L. is a perennial shrub preferring humid fields and meadows overall in Europe. The 50 to 100 cm-high erect stem is covered by trichomes. It has elliptical leaves and pink flowers.[1] The synonym names are *Ononis hircina* Jacq. and *Ononis spinosa subsp. hircina* (Jacq.) Gams. In the Renaissance, it was used in the treatment of epilepsy,[2] but its most widespread use is to treat infections of the urinary tract and for skin diseases.[3] In ethnomedicinal reports, the decoction of the aerial part has been applied to liver and stomach disorders in the human and veterinary medicine, as well.[4,5]

In the aerial parts, flavonoids and hydroxycinnamic acids were characterized and determined quantitatively using UHPLC-ESI-Q-TOF-MS.[5] Sichinava et al. isolated oxycoumarins, scopoletin and scopolin from the plant.[6] The distribution of phytosterols and triterpene onocerin was investigated in the aerial parts and the roots of *O. arvensis* by GLC-MS.[7] Only a limited number of papers can be found dealing with the chemical composition of the roots. Horoejsi et al. isolated and characterized the lectins of *O. arvensis* root.[8] The isoflavonoid glucoside ononin and the dihydroisoflavonoid onogenin were isolated from the roots, and the structure of onogenin was elucidated by NMR spectroscopy[9]; however, there is a lack of the detailed investigation of the isoflavonoid profile of the plant.

For screening numerous isoflavonoid derivatives, liquid chromatography coupled with tandem mass spectrometry is the most powerful tool regarding its selectivity and sensitivity. With the application of tandem mass spectrometry, the fragmentation patterns of isoflavonoid derivatives can be examined and compared. As isoflavonoids can be found in the form of glucosides, glucoside malonates, aglycones[10] and beta amino acid glucoside esters,[11] the similarity of their product ion spectra can be used to classify the derivatives with the same aglycone. In some cases, mass spectrometry on its own is not sufficient for the complete structural elucidation, so the application of NMR techniques is inevitable. To obtain the necessary amount of pure compound for NMR experiments, the most repeatable and reliable way is to use preparative HPLC.

Previous studies on the composition of *O. arvensis* aerial parts and root dealt in depth only with selected compounds, and the structural analysis and characterization of other derivatives were missed. Therefore, the aim of this study is to systematically identify the isoflavonoid profile of the aqueous-methanolic extract of *O. arvensis* aerial parts and root by HPLC-ESI-MS/MS, UHPLC-ESI-FTMS/MS in positive ionization mode in conjunction with NMR.

2 | EXPERIMENTAL

2.1 | General and plant material

HPLC-grade methanol was obtained from Fisher Scientific (Loughborough, UK). Methanol-d_4, for NMR measurements, was purchased from Sigma-Aldrich (Steinheim, Germany). Purified water prepared by Millipore Milli-Q equipment (Billerica, MA, USA) was used throughout the study. Calycosin, homoproline, and homopipecolic acid were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical grade. *O. arvensis* was collected near Beregújfalu (location: N 48°17′21.1″, E 22°48′08.7″—Beregszászi járás, Ukraine, July 2017). Voucher specimens were deposited in the Department of Pharmacognosy, Semmelweis University, Budapest with voucher number 170727-OnArv02. The roots and the aerial parts of the plant were separated. The roots were washed to remove soil, and the dried roots were ground. The aerial parts were ground without further separation of leaves and stems.

2.2 | Preparation of analytical sample

From the ground plant material, 0.500 g was mixed with 30 mL of 70% aqueous methanol and extracted in ultrasonic bath for 10 minutes on 25°C. After filtration, the sample was dried under vacuum with rotary evaporator (60°C, Heidolph Instruments, Laborata 4000, Schwabach, Germany). The resulting residue was redissolved in 2 mL of 70% aqueous methanol and filtered through 0.22 μm PTFE filter (Nantong FilterBio Membrane Co., Ltd; Nantong City, Jiangsu P. R China). For the hydrolyzed sample, 1 mL of the analytical sample was mixed with 1 mL of concentrated ammonia and evaporated to dryness with rotary evaporator set to 60°C. The residue was mixed with 2 mL of purified water, and the liquid was passed through the same PTFE filter.

2.3 | HPLC-ESI-MS/MS conditions

For chromatographic separation and mass spectral analysis, an Agilent 1100 HPLC system (degasser, binary gradient pump, autosampler, column thermostat, and diode array detector) was used hyphenated with an Agilent 6410 Triple Quad LC/MS system equipped with ESI ion source (Agilent Technologies, Santa Clara, CA, USA). The HPLC separation of the root and aerial part extracts was attained on a Zorbax SB-C18 Solvent Saver Plus (3.5 μm) reversed phase column (150 × 3.0 mm i.d; Agilent Technologies, Santa Clara, CA, USA). Mobile phase consisted of 0.3% v/v formic acid (A) and methanol (B). The following gradient program was applied: 0.0 minutes, 29% B; 32.0 minutes, 80% B; 34 minutes, 100% B; 37 minutes, 100% B; 42.0 minutes, 29% B. Solvent flow rate was 0.4 mL/min, and the column temperature was set to 25°C. The injection volume was 2 μL. Nitrogen was applied as drying gas at the temperature of 350°C at 9 L/min; the nebulizer pressure was 45 psi. Full scan mass spectra were recorded in positive ionization mode in the range of *m/z* 80 to 1500. For collision induced dissociation (CID), the collision energy varied between 10 and 40 eV. As collision gas, high purity nitrogen was used. The fragmentor voltage was set to 80 V, and the capillary voltage was 3500 V. Product ion mass spectra were recorded in positive ionization mode in the range of *m/z* 50 to 600.

The hydrolyzed sample was analyzed using the same HPLC-MS/MS apparatus equipped with a Zorbax NH_2 normal phase column (150 × 4.6 mm i.d; 5 μm). Mobile phase consisted of 20 mM ammonium formate buffer (pH = 4) (A) and acetonitrile (B). Isocratic mode was applied with 80% B at 1 mL/min flow rate and at 25°C. The injection volume was 5 μL. Nitrogen was applied as drying gas at the temperature of 300°C at 6 L/min; the nebulizer pressure was 15 psi. For registering the chromatogram, selective ion monitoring mode was chosen at *m/z* 130 (homoproline) and *m/z* 144 (homopipecolic acid). For CID, the collision energy varied between 10 and 30 eV. As collision gas high purity nitrogen was used. The fragmentor voltage was set to 120 V, and the capillary voltage was 4000 V. Product ion mass spectra were recorded in positive ion mode in the range of *m/z* 50 to 200.

2.4 | UPLC-ESI-Orbitrap-MS/MS conditions

For obtaining high resolution mass spectrometric data of the root and aerial part extracts, a Dionex Ultimate 3000 UHPLC system (3000RS diode array detector, TCC-3000RS column thermostat, HPG-3400RS pump, SRD-3400 solvent rack degasser, WPS-3000TRS autosampler) was used hyphenated with a Orbitrap Q Exactive Focus Mass Spectrometer equipped with electrospray ionization (Thermo Fischer Scientific, Waltham, MA, USA). The column and the HPLC method were the same as the ones used with the non-hydrolyzed analytical samples. The electrospray ionization source was operated in positive ionization mode, and operation parameters were optimized automatically using the built-in software. The working parameters were as follows: spray voltage, 3500 V; capillary temperature 256.25°C; sheath gas (N_2), 47.5°C; auxillary gas (N_2), 11.25 arbitrary units; spare gas (N_2), and 2.25 arbitrary units. The resolution of the full scan was of 70 000, and the scanning range was between 120 and 1000 *m/z* units.

The most intense ions detected in full scan spectrum were selected for data-dependent MS/MS scan at a resolving power of 35 000, in the range of 50 to 1000 *m/z* units. Parent ions were fragmented with normalized collision energy of 10%, 30%, and 45%.

2.5 | NMR conditions

All NMR experiments were carried out on a 600 MHz Varian DDR NMR spectrometer equipped with a 5 mm inverse-detection gradient (IDPFG) probehead. Standard pulse sequences and processing routines available in VnmrJ 3.2C/Chempack 5.1 were used for structure identifications. The complete resonance assignments were established from direct ^{1}H-^{13}C, long-range ^{1}H-^{13}C, and scalar spin-spin connectivities using 1D ^{1}H, ^{13}C, ^{1}H-^{1}H gCOSY, ^{1}H-^{1}H NOESY, ^{1}H-^{1}H ROESY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C gHSQCAD (J = 140 Hz), and ^{1}H-^{13}C gHMBCAD (J = 8 Hz and 12 Hz) experiments, respectively. The probe temperature was maintained at 298 K, and standard 5 mm NMR tubes were used. The ^{1}H and ^{13}C chemical shifts were referenced to the residual solvent signal δ_H = 3.310 ppm and δ_C = 49.00 ppm, respectively.

2.6 | Isolation of licoagroside B

Using ultrasonic bath on room temperature, 20.0 g ground root was extracted with 200 mL of 70% methanol twice. After filtration, the extract was dried under reduced pressure. The residue was redissolved in water, and 10 mL of acetone was added to remove saccharides. The precipitate was filtered, and the liquid phase was dried. The residue was redissolved in 10 mL of water and passed through Supelclean SPE LC-18 columns (500 mg, 3 mL; Supelco, Bellefonte, PA, USA). After air drying the cartridges, 3 mL of 50% methanol was used to elute glycosides, then 6 mL pure methanol was applied to achieve complete elution of isoflavonoids. The weights of the first and second eluates were 274 and 171 mg, respectively. The 50% methanol fraction was redissolved in 2 mL of water and filtered through 0.22 µm PTFE filter before subjected to preparative HPLC. For fractionation, a Hanbon Newstyle NP7000 HPLC system with a Hanbon Newstyle NP3000 UV detector (Hanbon Sci. & Tech. CO. Jiangsu, China) equipped with a Gemini C18 reversed phase column (150 × 21.2 mm i.d; 5 µm, Phenomenex Inc; Torrance, CA, USA) was used. Eluents consisted of 0.3% v/v acetic acid (A) and methanol (B). Gradient elution was used with a 10 mL/min flow rate and a solvent system using 10% B at 0 minutes, 40% B in 10 minutes, 100% B in 15 minutes, and 10% B in 25 minutes. This method has not been optimized in terms of performance parameters as it only served for isolation purposes. Licoagroside B eluted at 11.41 minutes, the obtained fraction was reinjected for further purification. Finally, 8.9-mg licoagroside B was yielded in high purity

2.7 | Isolation of but-2-enolide aglycones

From the same plant material, 30.0 g was mixed with 200 of mL water for 48 hours to activate the plant's indigenous glucosidase enzymes. After filtration, the drug was extracted twice with 200 mL of 70% methanol using ultrasonic bath at room temperature. The extract was dried under reduced pressure and redissolved in water. The saccharides were precipitated with the same method as mentioned above. The total weight of the extract was 835 mg and was redissolved in 10 mL of water and filtered through 0.22 µm PTFE filter before subjected to the same preparative HPLC system. The chosen chromatographic conditions fulfilled the criteria of isolation but were not optimized in terms of performance parameters. Eluents consisted of 0.3% v/v acetic acid (A) and methanol (B). Gradient elution was used with a 10 mL/min flow rate and solvent system with 50% B at 0 minutes, 50% B in 10 minutes, 100% B in 15 minutes, and 50% B in 20 minutes. Puerol A eluted at 8.40 minutes, while clitorienolactone B eluted at 12.25 minutes. Clitorienolactone B was reinjected for further purification with isocratic 25% acetonitrile as solvent B. The yields were 4.8 mg for puerol A and 3.1 mg for clitorienolactone B, respectively.

2.8 | Isolation of but-2-enolide glycosides and calycosin D glycosides

100 gram powdered drug was extracted by 400 mL of 70% aqueous methanol twice. After filtration, the liquid phase was dried under reduced pressure at 60°C. The residue was dissolved in water to gain a viscous solution of 500 mg/mL concentration. This sample was purified using a CombiFlash NextGen 300+ (Teledyne ISCO, Lincoln, USA) equipped with a RediSep Rf Gold C18 column (150 g). As eluents, methanol (solvent B) and 0.3% acetic acid (solvent A) were used with the following gradient program: 0 minutes 30% B, 20 minutes 50% B, 25 minutes 100% B, and 30 minutes 100% B. The flow was set to 60 mL/min and 16 mL fractions were collected. Fractions 23 to 27, 38 to 41, and 49 to 53 were unified and further purified by the same preparative HPLC system using isocratic 25% acetonitrile as eluent with 10 mL/min flow. Fractions 23 to 27 yielded 15.4 mg calycosin D glucoside. From fractions 38 to 41, puerol A 2′-*O*-glucoside was isolated (eluted at 7.2 minutes, 63.2 mg) along with clitorienolactone B 4′-*O*-glucoside (eluted at 8.6 minutes). Clitorienolactone B 4′-*O*-glucoside was further purified on a Luna C18(2) 100 A (5 µm) reversed phase column (150 × 10.00 mm i.d; Phenomenex, Inc; USA) using isocratic 25% acetonitrile and 2 mL/min flow, yielding 2.3 mg. Calycosin D 6″-O-glucoside malonate was isolated from fractions 49 to 53 eluting at 11.3 min (1.1 mg).

3 | RESULTS AND DISCUSSION

In the aqueous-methanolic extract of *O. arvensis* aerial parts and roots altogether, 47 compounds were described (Figure 1). Isoflavonoids, dihydroisoflavonoids, and pterocarpans were characterized in the form of glucosides, glucoside malonates, aglycones, and esters of homopipecolic acid besides several new compounds. Moreover, the glucosides of some special phenolic compounds with their aglycones (puerol A and clitorienolactone B) and a maltol glucoside derivative (licoagroside B) were also identified in the samples (see Table 1). In the case of nitrogen containing compounds, diastereomeric splitting could be observed depending on the type of the aglycone and the

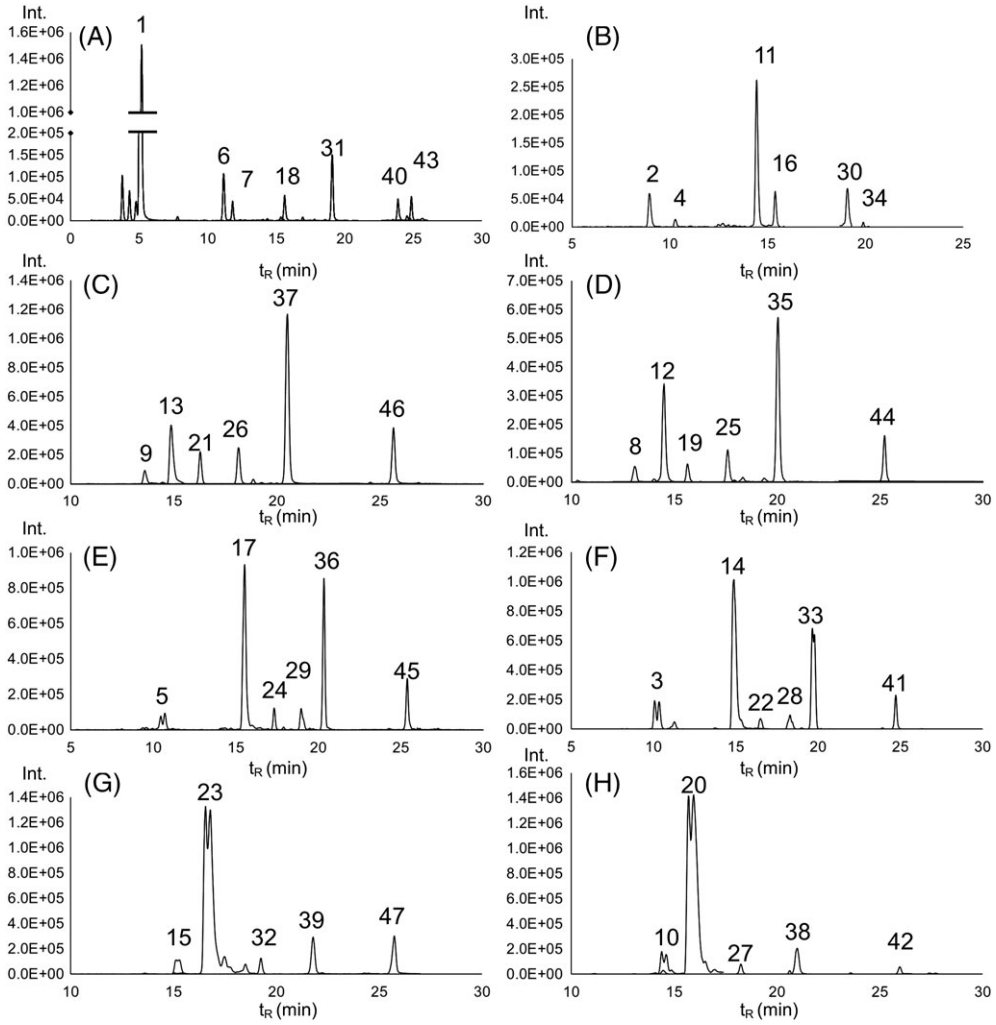


FIGURE 1 The extracted ion chromatograms of the described compounds with various aglycones from *O. arvensis* root aqueous-methanolic extract. A, Licoagroside B (**1**—433.1338), puerol derivatives (**6**—461.1435, **7**—475.1593, **18**—299.0909, **31**—313.1066), 2′-methoxy isoflavonoids (**39**—313.0703, **42**—299.0916); B, Calycosin D derivatives (**2**—447.1281, **11**—533.1295, **30**—285.0754) and calycosin derivatives (**4**—447.1299, **16**—533.1309, **34**—285.0752); C, Formononetin derivatives (**9**—542.2020, **13**—556.2177, **21**—431.1345, **26**—517.1343, **36**—517.1353, **45**—269.0803); D, Pseudobaptigenin derivatives (**8**—556.1812, **12**—570.1967, **19**—445.1124, **25**—531.1125, **35**—531.1132, **44**—283.0596); E, Sativanone derivatives (**5**—574.2274, **17**—588.2234, **24**—463.1591, **29**—549.1600, **36**—549.1595, **45**—301.1061); F, Onogenin derivatives (**3**—588.2072, **14**—602.2231, **22**—477.1382, **28**—563.1387, **33**—563.1382, **41**—315.0858); G, Medicarpin derivatives (**15**—544.2166, **23**—558.2326, **32**—433.1487, **39**—519.1488, **47**—271.0989); H, Maackiain derivatives (**10**—558.1968, **20**—572.2122, **27**—447.1272, **38**—533.1275, **42**—285.0750)

retention time (Figure 1). Comparing the metabolic profile of the aerial parts and the roots, a significant difference emerges between the amounts of dihydroisoflavonoid compounds (onogenin and sativanone). These derivatives could be found in the aerial parts only in trace quantities, while they were quite abundant in the root extracts, indicating a divergence in biosynthesis (Table 1).

3.1 | Identification of licoagroside B

The *m/z* value of the pseudo-molecular ion in positive ionization mode of compound **1** (Figure 1A) was 433.1338, and its molecular formula calculated on the basis of HR-MS experiments corresponds to $C_{18}H_{24}O_{12}$ (see Table 1). Investigating the fragmentation pattern of this precursor ion, only two fragment ions at *m/z* 145.0493 ($C_6H_9O_4$) and *m/z* 127.0390 ($C_6H_7O_3$) could be observed, contrary to the rich fragmentation profile and retro Diels-Alder (rDA) cleavage of isoflavonoid derivatives.[12] Based on these results, peak **1** was tentatively identified as licoagroside B, the 3-hydroxy-3-methyl-glutarate ester of maltol glucoside. The fragment at *m/z* 127.0390 could result from the cleavage of the maltol ring together with the anomeric *O* atom, while the fragment at *m/z* 145.0493 could be assigned to the hydroxy-methyl-glutaric acid residue (Figure 2). The results of the NMR experiments verified that compound **1** was licoagroside B (Table S1), and the obtained resonances showed perfect correlation with the ones reported by Li et al.[13] Licoagroside B was only identified in the hairy root cultures of *Glycyrrhiza glabra* L. till date. However, licoagroside B is present in high quantity in *O. arvensis*, showing that this compound is a characteristic metabolite of *Ononis* species.

TABLE 1 The identified compounds and their high-resolution MS and MS/MS data of *O. arvensis* root and aerial parts

No	Rt *min*	$[M + H]^+$ *m/z*	Delta *ppm*	Protonated Formula	Aglycone *m/z*	MS/MS Fragment Ions (Protonated Formula) *m/z*	Identification	Roots	Aerial Parts
1	5.08	433.1338	−0.58	$C_{18}H_{25}O_{12}$		145.0493 ($C_9H_9O_4$), 127.0390 ($C_6H_7O_3$)	Licoagroside B	+	+
2	8.95	447.1281	−1.06	$C_{22}H_{23}O_{10}$	285.0753	270.0521 ($C_{15}H_{10}O_5$), 253.0491 ($C_{15}H_9O_4$), 225.0542 ($C_{14}H_9O_3$), 213.0542 ($C_{13}H_9O_3$), 197.0594 ($C_{13}H_9O_2$)	Calycosin D 7-*O*-β-D-glucoside	+	+
3	10.07	588.2072	−0.60	$C_{29}H_{34}NO_{12}$	315.0857	287.0912 ($C_{16}H_{15}O_5$), 274.1284 ($C_{12}H_{20}NO_6$), 177.0545 ($C_{10}H_9O_3$), 163.0383 ($C_9H_7O_3$), 130.0861 ($C_6H_{12}NO_2$), 70.0658 (C_4H_8N)	Onogenin 7-*O*-β-D-glucoside 6″-pyrrolidine 2-acetate	+	-
4	10.29	447.1299	2.97	$C_{22}H_{23}O_{10}$	285.0752	270.0517 ($C_{15}H_{10}O_5$), 253.0486 ($C_{15}H_9O_4$), 225.0538 ($C_{14}H_9O_3$), 213.0543 ($C_{13}H_9O_3$), 197.0593 ($C_{13}H_9O_2$)	Calycosin 7-*O*-β-D-glucoside	+	+
5	10.71	574.2274	−1.55	$C_{29}H_{36}NO_{11}$	301.1064	283.0598 ($C_{16}H_{11}O_5$), 274.1280 ($C_{12}H_{20}NO_6$), 163.0389 ($C_9H_7O_3$), 130.0862 ($C_6H_{12}NO_2$), 70.0655 (C_4H_8N)	Sativanone 7-*O*-β-D-glucoside 6″-pyrrolidine 2-acetate	+	-
6	11.17	461.1435	−1.57	$C_{23}H_{25}O_{10}$	299.0908	281.0802 ($C_{17}H_{13}O_4$), 253.0854 ($C_{16}H_{13}O_3$), 239.0698 ($C_{15}H_{11}O_3$), 193.0493 ($C_{10}H_9O_4$), 107.0495 (C_7H_7O)	Puerol A 2′-*O*-glucoside	+	+
7	11.82	475.1593	−1.21	$C_{24}H_{27}O_{10}$	313.1069	295.0960 ($C_{18}H_{15}O_4$), 267.1012 ($C_{17}H_{15}O_3$), 253.0855 ($C_{16}H_{13}O_3$), 207.0647 ($C_{11}H_{11}O_4$), 107.0495 (C_7H_7O)	Clitorienolactone B 4′-*O*- β-D-glucoside	+	+
8	13.07	556.1812	−0.25	$C_{28}H_{30}NO_{11}$	283.0789	274.1284 ($C_{12}H_{20}NO_6$), 70.0650 (C_4H_8N)	Pseudobaptigenin 7-*O*-β-D-glucoside 6″ -pyrrolidine 2-acetate	+	+
9	13.60	542.2020	0.13	$C_{28}H_{32}NO_{10}$	269.0804	274.1282 ($C_{12}H_{20}NO_6$), 70.0654 (C_4H_8N)	Formononetin 7-*O*-β-D-glucoside 6″ -pyrrolidine 2-acetate	+	+
10	14.39	558.1968	−0.335	$C_{28}H_{32}NO_{11}$	285.0754	274.1280 ($C_{12}H_{20}NO_6$), 175.0389 ($C_{10}H_7O_3$), 151.0388 ($C_8H_7O_3$), 70.0658 (C_4H_8N)	Maackiain 3-*O*-β-D-glucoside 6″-pyrrolidine 2-acetate	+	+
11	14.44	533.1295	1.00	$C_{25}H_{25}O_{13}$	285.0753	270.0518 ($C_{15}H_{10}O_5$), 253.0490 ($C_{15}H_9O_4$), 225.0542 ($C_{14}H_9O_3$), 213.0542 ($C_{13}H_9O_3$), 197.0597 ($C_{13}H_9O_2$)	Calycosin D 7-*O*-β-D-glucoside malonate	+	+
12	14.50	570.1967	−0.504	$C_{29}H_{32}NO_{11}$	283.0596	288.1434 ($C_{13}H_{22}NO_6$), 84.0814 ($C_5H_{10}N$)	Pseudobaptigenin 7-*O*-β-D-glucoside 6″-piperidine 2-acetate	+	+
13	14.87	556.2177	−0.04	$C_{29}H_{34}NO_{10}$	269.0801	288.1436 ($C_{13}H_{22}NO_6$), 144.1017 ($C_7H_{14}NO_2$), 84.0814 ($C_5H_{10}N$)	Formononetin 7-*O*-β-D-glucoside 6″-piperidine 2-acetate	+	+
14	14.89	602.2231	−0.17	$C_{30}H_{36}NO_{12}$	315.0855	288.1435 ($C_{13}H_{22}NO_6$), 177.0543 ($C_{10}H_9O_3$), 163.0387 ($C_9H_7O_3$), 144.1017 ($C_7H_{14}NO_2$), 135.0439 ($C_8H_7O_2$), 84.0814 ($C_5H_{10}N$)	Onogenin 7-*O*-β-D-glucoside 6″-piperidine 2-acetate	+	-
15	15.27	544.2166	−2.063	$C_{28}H_{34}NO_{10}$	271.0961	274.1281 ($C_{12}H_{20}NO_6$), 161.0594 ($C_{10}H_9O_2$), 137.0595 ($C_8H_9O_2$), 123.0441 ($C_7H_7O_2$), 70.0646 (C_4H_8N)	Medicarpin 3-*O*-β-D-glucoside 6″-pyrrolidine 2-acetate	+	+
16	15.39	533.1309	−1.40	$C_{25}H_{25}O_{13}$	285.0753	270.0518 ($C_{15}H_{10}O_5$), 253.0491 ($C_{15}H_9O_4$), 225.0541 ($C_{14}H_9O_3$), 213.0543 ($C_{13}H_9O_3$), 197.0594 ($C_{13}H_9O_2$)	Calycosin 7-*O*-β-D-glucoside malonate	+	+
17	15.53	588.2434	−0.91	$C_{30}H_{38}NO_{11}$	301.1064	288.1436 ($C_{13}H_{22}NO_6$), 273.1115 ($C_{16}H_{17}O_4$), 163.0387 ($C_9H_7O_3$), 144.1017 ($C_7H_{14}NO_2$), 135.0439 ($C_8H_7O_2$), 84.0814 ($C_5H_{10}N$)	Sativanone 7-*O*-β-D-glucoside 6″-piperidine 2-acetate	+	-
18	15.61	299.0909	−1.67	$C_{17}H_{15}O_5$		281.0804 ($C_{17}H_{13}O_4$), 253.0805 ($C_{16}H_{13}O_3$), 239.0699 ($C_{15}H_{11}O_3$), 193.0493 ($C_{10}H_9O_4$), 107.0495 (C_7H_7O)	Puerol A	+	+
19	15.65	445.1124	−1.18	$C_{22}H_{21}O_{10}$	283.0597	253.0491 ($C_{15}H_9O_4$), 225.0543 ($C_{14}H_9O_3$), 197.0595 ($C_{13}H_9O_2$), 169.0647 ($C_{12}H_9O$)	Pseudobaptigenin 7-*O*-β-D-glucoside	+	+
20	15.95	572.2122	−0.76	$C_{29}H_{34}NO_{11}$	285.0752	288.1436 ($C_{13}H_{22}NO_6$), 175.0387 ($C_{10}H_7O_3$), 151.0388 ($C_8H_7O_3$), 144.1017 ($C_7H_{14}NO_2$), 84.0814 ($C_5H_{10}N$)	Maackiain 3-*O*-β-D-glucoside 6″-piperidine 2-acetate	+	+

(Continues)

TABLE 1 (Continued)

No	Rt *min*	$[M + H]^+$ *m/z*	Delta *ppm*	Protonated Formula	Aglycone *m/z*	MS/MS Fragment Ions (Protonated Formula) *m/z*	Identification	Roots	Aerial Parts
21	16.28	431.1345	1.95	$C_{22}H_{23}O_9$	269.0805	254.0570 ($C_{15}H_{10}O_4$), 237.0543 ($C_{15}H_9O_3$), 226.0622 ($C_{14}H_{10}O_3$), 213.0907 ($C_{14}H_{13}O_2$), 118.0415 (C_8H_6O)	Formononetin 7-*O*-β-D-glucoside	+	+
22	16.51	477.1382	−1.97	$C_{23}H_{25}O_{11}$	315.0858	297.0753 ($C_{17}H_{13}O_5$), 287.0909 ($C_{16}H_{15}O_5$), 257.0805 ($C_{15}H_{13}O_4$), 229.0857 ($C_{14}H_{13}O_3$), 178.0623 ($C_{10}H_{10}O_3$), 163.0388 ($C_9H_7O_3$), 147.0439 ($C_9H_7O_2$), 135.0440 ($C_8H_7O_2$)	Onogenin 7-*O*-β-D-glucoside	+	-
23	16.55	558.2326	−1.384	$C_{29}H_{36}NO_{10}$	271.0959	288.1435 ($C_{13}H_{22}NO_6$), 161.0594 ($C_{10}H_9O_2$), 144.1017 ($C_7H_{14}NO_2$), 137.0595 ($C_8H_9O_2$), 123.0441 ($C_7H_7O_2$), 84.0814 ($C_5H_{10}N$)	Medicarpin 3-*O*-β-D-glucoside 6″-piperidine 2-acetate	+	+
24	17.33	463.1591	−1.67	$C_{23}H_{27}O_{10}$	301.1065	283. ($C_{16}H_{11}O_5$), 273.1116 ($C_{16}H_{17}O_4$), 177.1119 ($C_8H_{17}O_4$), 163.0388 ($C_9H_7O_3$), 135.0440 ($C_8H_7O_2$)	Sativanone 7-*O*-β-D-glucoside	+	-
25	17.59	531.1125	−1.54	$C_{25}H_{23}O_{13}$	283.0596	253.0490 ($C_{15}H_9O_4$), 225.0542 ($C_{14}H_9O_3$)	Pseudobaptigenin 7-*O*-β-D-glucoside 4″-malonate	+	+
26	18.14	517.1343	−0.48	$C_{25}H_{25}O_{12}$	269.0804	253.0483 ($C_{15}H_9O_4$)	Formononetin 7-*O*-β-D-glucoside 4″-malonate	+	+
27	18.23	447.1272	0.16	$C_{22}H_{23}O_{10}$	285.0751	175.0388 ($C_{10}H_7O_3$), 151.0388 ($C_8H_7O_3$), 123.0442 ($C_7H_7O_2$)	Maackiain 3-*O*-β-D-glucoside	+	+
28	18.31	563.1387	−1.48	$C_{26}H_{27}O_{14}$	315.0858	297.0753 ($C_{17}H_{13}O_5$), 287.0909 ($C_{16}H_{15}O_5$), 257.0804 ($C_{15}H_{13}O_4$), 229.0857 ($C_{14}H_{13}O_3$), 178.0623 (C10H10O3), 163.0388 ($C_9H_7O_3$), 147.0439 ($C_9H_7O_2$), 135.0440 ($C_8H_7O_2$)	Onogenin 7-*O*-β-D-glucoside 4″-malonate	+	-
29	18.97	549.1600	−0.49	$C_{26}H_{29}O_{13}$	301.1065	273.1117 ($C_{16}H_{17}O_4$), 177.1119 ($C_8H_{17}O_4$), 163.0388 ($C_9H_7O_3$), 135.0440 ($C_8H_7O_2$)	Sativanone 7-*O*-β-D-glucoside 4″-malonate	+	-
30	19.08	285.0754	1.23	$C_{16}H_{13}O_5$		270.0519 ($C_{15}H_{10}O_5$), 253.0490 ($C_{15}H_9O_4$), 225.0540 ($C_{14}H_9O_3$), 213.0543 ($C_{13}H_9O_3$), 197.0596 ($C_{13}H_9O_2$), 137.0232 ($C_7H_5O_3$)	Calycosin D	+	+
31	19.08	313.1066	−2.11	$C_{18}H_{17}O_5$		295.0961 ($C_{18}H_{15}O_4$), 267.1012 ($C_{17}H_{15}O_3$), 253.0856 ($C_{16}H_{13}O_3$), 207.0650 ($C_{11}H_{11}O_4$), 107.0495 (C_7H_7O)	Clitorienolactone B	+	+
32	19.27	433.1487	−1.41	$C_{22}H_{25}O_9$	271.0960	137.0596 ($C_8H_9O_2$)	Medicarpin 3-*O*-β-D-glucoside	+	+
33	19.68	563.1382	−2.37	$C_{26}H_{27}O_{14}$	315.0855	297.0752 ($C_{17}H_{13}O_5$), 287.0912 ($C_{16}H_{15}O_5$), 257.0803 ($C_{15}H_{13}O_4$), 229.0856 (C14H13O3), 178.0628 ($C_{10}H_{10}O_3$), 163.0387 ($C_9H_7O_3$), 147.0438 ($C_9H_7O_2$), 135.0439 ($C_8H_7O_2$)	Onogenin 7-*O*-β-D-glucoside 6″-malonate	+	-
34	19.90	285.0752		$C_{16}H_{13}O_5$		270.0519 ($C_{15}H_{10}O_5$), 253.0490 ($C_{15}H_9O_4$), 225.0542 ($C_{14}H_9O_3$), 213.0543 ($C_{13}H_9O_3$), 197.0592 ($C_{13}H_9O_2$),	Calycosin		
35	20.03	531.1132	−0.22	$C_{25}H_{23}O_{13}$	283.0596	253.0491 ($C_{15}H_9O_4$), 225.0543 ($C_{14}H_9O_3$), 197.0594 ($C_{13}H_9O_2$)	Pseudobaptigenin 7-*O*-β-D-glucoside 6″-malonate	+	+
36	20.35	549.1595	−1.40	$C_{26}H_{29}O_{13}$	301.1066	283.1001 ($C_{16}H_{11}O_5$), 273.1110 ($C_{16}H_{17}O_4$), 177.1144 ($C_8H_{17}O_4$), 163.0471 ($C_9H_7O_3$), 135.0455 ($C_8H_7O_2$)	Sativanone 7-*O*-β-D-glucoside 6″-malonate	+	-
37	20.51	517.1353	2.42	$C_{25}H_{25}O_{12}$	269.0803	254.0569 (C18H10O4), 237.0541 ($C_{15}H_9O_3$), 213.0906 ($C_{14}H_{13}O_2$)	Formononetin 7-*O*-β-D-glucoside 6″-malonate	+	+
38	20.98	533.1275	−2.75	$C_{25}H_{25}O_{13}$	285.0750	175.0387 ($C_{10}H_7O_3$), 151.0387 ($C_8H_7O_3$), 123.0441 ($C_7H_7O_2$)	Maackiain 3-*O*-β-D-glucoside 6″-malonate	+	+
39	21.77	519.1488	−1.74	$C_{25}H_{27}O_{12}$	271.0959	161.0959 ($C_{10}H_9O_2$), 137.0595 ($C_8H_9O_2$), 123.0441 ($C_7H_7O_2$)	Medicarpin 3-*O*-β-D-glucoside 6″-malonate	+	+

(Continues)

TABLE 1 (Continued)

No	Rt *min*	[M + H]$^+$ *m/z*	Delta *ppm*	Protonated Formula	Aglycone *m/z*	MS/MS Fragment Ions (Protonated Formula) *m/z*	Identification	Roots	Aerial Parts
40	23.88	313.0703	−1.17	$C_{16}H_{13}O_6$		298.0469 ($C_{16}H_{10}O_6$), 283.0598 ($C_{16}H_{11}O_5$), 281.0440 ($C_{16}H_9O_5$), 268.0362 ($C_{15}H_8O_5$), 255.0647 ($C_{15}H_{11}O_4$), 240.0413 ($C_{14}H_8O_4$), 212.0465 ($C_{13}H_8O_3$), 162.0310 ($C_9H_6O_3$), 151.0388 ($C_8H_7O_3$)	Cuneatin	+	+
41	24.72	315.0858	−1.63	$C_{17}H_{15}O_6$		297.0753 ($C_{17}H_{13}O_5$), 287.0903 ($C_{16}H_{15}O_5$), 257.0804 ($C_{15}H_{13}O_4$), 229.0857 (C14H13O3), 178.0623 ($C_{10}H_{10}O_3$), 163.0388 ($C_9H_7O_3$), 147.0435 ($C_9H_7O_2$), 135.0440 ($C_8H_7O_2$)	Onogenin	+	-
42	24.81	285.0750	0.88	$C_{16}H_{13}O_5$		175.0388 ($C_{10}H_7O_3$), 151.0388 ($C_8H_7O_3$), 123.0442 ($C_7H_7O_2$)	Maackiain	+	+
43	24.86	299.0916	0.67	$C_{17}H_{15}O_5$		284.0658 ($C_{16}H_{12}O_5$), 267.0649 ($C_{16}H_{11}O_4$), 252.0412 ($C_{15}H_8O_4$), 243.1014 ($C_{15}H_{15}O_3$), 213.0551 ($C_{13}H_9O_3$), 163.0387 ($C_9H_7O_3$), 148.0517 ($C_9H_8O_2$), 137.0596 ($C_8H_9O_2$)	2′-methoxy formononetin	+	+
44	25.22	283.0596	−1.77	$C_{16}H_{11}O_5$		253.0491 ($C_{15}H_9O_4$), 225.0543 ($C_{14}H_9O_3$), 197.0595 ($C_{13}H_9O_2$)	Pseudobaptigenin	+	+
45	25.37	301.1061	−3.16	$C_{17}H_{17}O_5$		273.1116 ($C_{16}H_{17}O_4$), 177.1272 ($C_8H_{17}O_4$), 163.0388 ($C_9H_7O_3$), 151.0388 ($C_8H_7O_3$), 135.0440 ($C_8H_7O_2$), 107.0495 (C_7H_7O)	Sativanone	+	-
46	25.65	269.0803	−1.99	$C_{16}H_{13}O_4$		253.0490 ($C_{15}H_9O_4$), 237.0541 ($C_{15}H_9O_3$), 226.0623 ($C_{14}H_{10}O_3$), 225.0542 ($C_{14}H_9O_3$), 213.0906 ($C_{14}H_{13}O_2$), 197.0594 ($C_{13}H_9O_2$)	Formononetin	+	+
47	25.74	271.0959	1.43	$C_{16}H_{15}O_4$		161.0595 ($C_{10}H_9O_2$), 137.0595 ($C_8H_9O_2$), 123.0441 ($C_7H_7O_2$)	Medicarpin	+	+

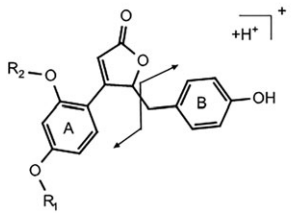
FIGURE 2 Proposed fragmentation profile of licoagroside B in positive ionization mode at 10 eV collision energy

3.2 | Identification of but-2-enolides

Peak **18** showed a protonated pseudo-molecular ion at *m/z* 299.0908, and its fragmentation pattern was identical with that of peak **6** bearing precursor ion at *m/z* 461.1435 (Figure 1A). Based on the protonated molecular formulas ($C_{17}H_{15}O_5$ and $C_{23}H_{25}O_{10}$), these structures were putatively identified as puerol A and its 2′-*O*-glucoside (Table 1). In their MS/MS spectra, two main fragmentation pathways could be observed: the cleavage of the whole molecule to A and B-ring and the neutral loss of small units, as CO and C_2H_2O.

Applying the same fragmentation pattern described above, **7** and **31** (Figure 1A) were assigned as the methylated derivative of puerol A and its *O*-glucoside, respectively. Between the fragment ions in the spectra of **18** and **31**, a difference of 14 Da could be observed in all cases (Table 1), except for the ion at *m/z* 107.0495 (C_7H_7O) corresponding to the B-ring (Figure 3). These fragments indicate that a methyl substituent on the A-ring is responsible for the 14 Da shifts of the fragments (Figure 3).

The nomenclature and structural identification of puerol derivatives are rather tangled in previous interpretations; naming and structures are briefly discussed below and summarized in Figure S8. Firstly, Kinjo et al. isolated pueroside A and B in 1985 and identified them as diglycosides of a ζ-lactone.[14] Shirataki et al. isolated sophoraside A (monoglycoside) and two aglycones, puerol A, and its 4′-*O*-methylated form, puerol B, possessing the same ζ-lactone structure,[15] and Barrero et al. identified specionin and its glucoside speciozide A with ζ-lactone structure in *O. speciosa*.[16] In their later work, Nohara et al.[17] described that pueroside A and B and sophoraside A were actually γ-lactones, in contrary to previous works. Kirmizgül et al.[18] isolated spinonin from *O. spinosa*, which is a monoglycoside and its structure would correspond to puerol A 2′-*O*-glucoside. Nevertheless, the authors drove to the conclusion that spinonin contained a 2,3-dihydro-3-oxofurane ring instead of a 2,3-dihydro-2-oxofurane, like the puerol derivatives. Puerol A and its 4′-*O*-methylated form, puerol B, along with their 2′-*O*-glucosides were isolated from *O. angustissima* L. by Ghribi et al.[19] but another *O*-methylated derivative of puerol A (clitorienolactone B) was isolated from *O. spinosa* by Addotey et al.[20] (See Figure S8). Puerol B and clitorienolactone differ only in the position of a methyl substitution. In puerol B, the methylation occurs at



R_1	R_2	Compound
H	H	Puerol A
H	glucose	Puerol A 2"-*O*-glucoside
CH_3	H	Puerol B
CH_3	glucose	Puerol B 2"-*O*-glucoside
H	CH_3	Clitorienolactone B
glucose	CH_3	Clitorienolactone B 4'-*O*-glucoside

FIGURE 3
The structure of puerol derivatives

the *para* OH group of A-ring position, whereas in clitorienolactone B it is in the *ortho* position. Since the HR-MS/MS investigations alone could not solve the exact location of atoms in the middle ring and the methyl group, peaks **6**, **7**, **18**, and **31** were isolated and subjected to NMR experiments in order to clarify the structures. Based on the NMR results, the isolated compounds contained a 2,3-dihydro-2-oxofurane ring. The most decisive element was the chemical shift of the carbonyl atoms, as they exhibited chemical shifts at 176.8 and 177.2 ppm which are characteristic for unsaturated γ-lactones,[21] whereas 2,3-dihydro-3-oxofurane rings possess chemical shift above 200 ppm.[22] The glucose moiety of compound **6** joined puerol A through the 2′-OH group, in *ortho* position. Based on the NOESY spectrum, the methyl group of compound **31** is located in *ortho* position, too, so that this compound was identified as clitorienolactone B. The NOESY spectrum of compound **7** revealed that the glucose moiety is linked through the 4′ OH group, in *para* position. To the best of our knowledge, this glucoside is characterized for the first time.

3.3 | Identification of isoflavonoids

Peak **21**, **26**, **37**, and **46** (Figure 1C) provided $[M + H]^+$ ions at *m/z* 431.1345 ($C_{22}H_{23}O_9$), 517.1343 ($C_{25}H_{25}O_{12}$), 517.1353 ($C_{25}H_{25}O_{12}$), and 269.0803 ($C_{16}H_{13}O_4$), respectively, but they did not differ in their MS/MS spectra, meaning that they are the derivatives of the same aglycone. The fragmentation of these ions gave rise to *m/z* 254.0570 ($C_{15}H_{10}O_4$) ion, owing to the radical cleavage of $CH_3^\bullet$ and *m/z* 237.0543 ($C_{15}H_9O_3$), deriving from the loss of a CH_3OH unit, verifying the presence of a methoxy group. The ion at *m/z* 213.0907 ($C_{14}H_{13}O_2$) is a result of the loss of two CO units which is characteristic for isoflavonoid aglycones.[23] Fragment ion at *m/z* 118.0415 (C_8H_6O) refers to the ion containing the B-ring resulting from the rDA fragmentation (Figure 4A). Although, the intact B-ring with the methoxy group at *m/z* 133.0648 (C_9H_9O) is barely detectable, the rDA fragment losing the $CH_3^\bullet$ radical at *m/z* 118.0415 is much more intense (Table 1). Regarding this information, the aglycone was tentatively identified as formononetin. The neutral loss of 162.0540 ($C_6H_{10}O_5$) Da of peak **21** corresponded to the loss of a hexose moiety. Taking into account, that isoflavonoids form glycosides with glucose in the vast majority of cases,[24] the peak was assigned as formononetin 7-*O*-β-D-glucoside or ononin. Peaks **26** and **37** showed the same quasi-molecular ion and fragmentation spectra, and a neutral loss of 248.0550 Da ($C_9H_{12}O_8$); therefore, they were attributed as 7-*O*-β-D-glucoside malonates of formononetin. Based on the significant difference in their quantity and retention times (Figure 1C), the firstly eluting minor derivative was tentatively identified as 4″-malonate (**26**) and the later eluting major molecule as 6″-malonate (**37**).[25]

With protonated pseudo-molecular ions at *m/z* 447.1281 ($C_{22}H_{23}O_{10}$) and 447.1299 ($C_{22}H_{23}O_{10}$), 533.1295 ($C_{25}H_{25}O_{13}$) and 533.1309 ($C_{25}H_{25}O_{13}$), 285.0753 ($C_{16}H_{13}O_5$) and 285.0752 ($C_{16}H_{13}O_5$), two sets of glucosides, glucoside malonates and aglycones were observed, all sharing identical fragmentation pattern. The same neutral losses could be detected as in the case of formononetin, namely the loss of a $CH_3^\bullet$ and a CH_3OH, which are a result of a methoxy substitution. These cleavages could be combined with the loss of two CO moieties, resulting in ions at *m/z* 270.0521 ($C_{15}H_{10}O_5$) $[M + H\text{-}CH_3^\bullet]^+$, 253.0491 ($C_{15}H_9O_4$) $[M + H\text{-}CH_3OH]^+$, 225.0542 ($C_{14}H_9O_3$) $[M + H\text{-}CH_3OH\text{-}CO]^+$, 213.0542 ($C_{13}H_9O_3$) $[M + H\text{-}CH_4OH\text{-}CO\text{-}CO]^+$, 197.0594 ($C_{13}H_9O_2$) $[M + H\text{-}CH_3OH\text{-}CO\text{-}CO]^+$ (Table 1). The ion at *m/z* 137.0232 ($C_7H_5O_3$) was presumed to be the rDA fragment containing the A-ring, demonstrating that it could not bear any other substituents but the hydroxy group at C7 (Figure 4A). As a result, these molecules were tentatively identified as structural isomers differing only in the position of the hydroxy and methoxy groups of the B-ring. As even the ratios of the fragment ions were not significantly different, the two sets were not distinguishable relying only on mass spectrometry data. Since the firstly eluting peaks of the pairs were always higher in relative quantity (see Figure 1), it was presumed that peaks **2**, **11**, and **30** have the same aglycone, and **4**, **16**, and **34** another one. The identity of the aglycones was investigated using calycosin as standard substance and its retention time and MS/MS spectrum matched with the later eluting peak (**34**). As Addotey et al. isolated calycosin D from *O. spinosa*,[20] which differ only in the position of a methyl group (see Figure 7), it

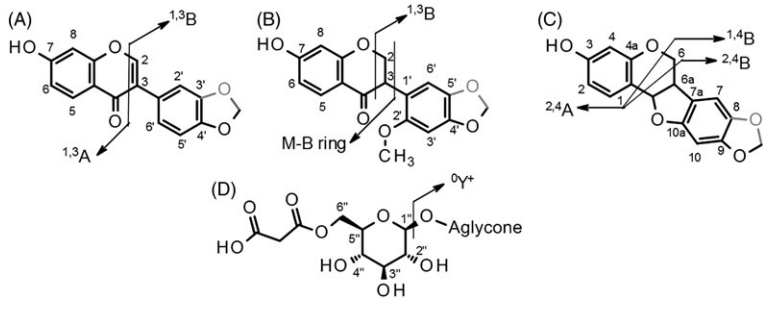


FIGURE 4 Schematic structures and main proposed fragmentation patterns of *Ononis* isoflavonoids (A), pterocarpans (B), dihydroisoflavonoids (C) and their glycosides (D)

was hypothesized that the major peaks (**4**, **16**, and **34**) are the derivatives of this molecule. To verify this hypothesis, the firstly eluting relative larger peaks (**2** and **11**) were isolated and investigated by NMR. Based on the NOESY spectra, they were the 7-*O*-glucoside and 7-*O*-glucoside 6″-*O*-malonate of calycosin D, so that peaks **4** and **16** were tentatively identified as the 7-*O*-glucoside and 7-*O*-glucoside 6″-*O*-malonate of calycosin, respectively. Peaks **19**, **25**, and **35** (Figure 1D) had a pseudo-molecular ion at 445.1124 ($C_{22}H_{21}O_{10}$), 531.1125 ($C_{25}H_{23}O_{13}$) and 531.1132 ($C_{25}H_{23}O_{13}$) and each provided the same fragment ions with peak **44** at *m/z* 283.0596 ($C_{16}H_{11}O_5$). The fragments, which were formed by the loss of a CH_3OH unit from the protonated calycosin ion (*m/z* 253.0491, 225.0543, 197.0595), could be observed with these molecules as well. As the protonated molecular formula of calycosin contains two hydrogens more ($C_{16}H_{13}O_5$) than the aglycone of these molecules ($C_{16}H_{11}O_5$) (Table 1), the cause of the same fragment ions is the neutral loss of CH_2O unit instead of a CH_3OH unit. Considering these results, the peaks were tentatively identified as the 7-*O*-β-D-glucoside (**19**), 4″ and 6″-malonates (**25** and **35**), and the aglycone of pseudobaptigenin (**44**) (Figure 7).

3.4 | Identification of pterocarpans

Peaks **32** and **39** (Figure 1G) had a pseudo-molecular ion at *m/z* 433.1487 ($C_{22}H_{25}O_9$) and 519.1488 ($C_{25}H_{27}O_{12}$) and showed a common aglycone fragment at *m/z* 271.0959 ($C_{16}H_{15}O_4$) with **47**. Their MS/MS fragments could be detected at *m/z* 161.0595 ($C_{10}H_9O_2$), 137.0595 ($C_8H_9O_2$), and 123.0441 ($C_7H_7O_2$) (Table 1). These results were in concordance with the fragmentation pattern of the pterocarpan medicarpin 3-*O*-β-D-glucoside (**32**), medicarpin 3-*O*-β-D-glucoside 6″-malonate (**39**), and medicarpin aglycone (**47**) (Figure 7). The ion at *m/z* 161.0595 is corresponding to the loss of a $C_6H_6O_2$ ($[^{1,4}A]^+$) unit, which can be a consequence of the cleavage of the bonds 1 and 4, whereas the ions at *m/z* 123.0441 and 137.0595 are the products of the cleavage of bond 2 and 4, resulting $[^{2,4}A]^+$ and $[^{2,4}B]^+$, respectively (Figure 4C). Peaks **27**, **38**, and **42** (Figure 1H) showed similar fragmentation pattern to those of medicarpin; however, a 14-Da difference (+O-2H) could be observed in the case of the quasi-molecular ions and the fragment ions containing the B-ring, namely at *m/z* 175.0388 ($C_{10}H_7O_3$) [M + H- $C_6H_6O_2$]$^+$ and 151.0388 ($C_8H_7O_3$) $[^{2,4}B]^+$. Based on this fragmentation pattern and analogy with medicarpin, these ions could be tentatively identified as maackiain 3-*O*-β-D-glucoside (**27**), maackiain 3-*O*-β-D-glucoside 6″-malonate (**38**), and maackiain (**42**) (Figure 7). As the two molecules differ only in the substitution of the B-ring, the fragment originating from the A-ring is the same at *m/z* 123.0442 $[^{2,4}A]^+$.

3.5 | Identification of dihydroisoflavonoids

Peak **24**, **29**, **36**, and **45** (Figure 1E) had the quasi-molecular ions at *m/z* 463.1591 ($C_{23}H_{27}O_{10}$), 549.1600, 549.1595 ($C_{26}H_{29}O_{13}$), and 301.1061 ($C_{17}H_{17}O_5$), and their fragmentation pattern significantly differed from those of the isoflavones. The MS/MS of these ions produced *m/z* 283.0965 ($C_{17}H_{15}O_4$) and 273.1119 ($C_{16}H_{17}O_4$) ions, corresponding to the initial ejection of a H_2O or a CO unit (Table 1). The two intense fragment ions at *m/z* 163.0388 ($C_9H_7O_3$) and 135.0440 ($C_8H_7O_2$) could be attributed to [M + H-B-ring]$^+$ and [M + H-B-ring-CO]$^+$ (Figure 4B). This typical fragmentation pattern was in agreement with our previous results[10]; consequently, the peaks were tentatively identified as the 7-*O*-glucoside (**24**), the 4″ and 6″-*O*-glucoside malonates (**29** and **36**), and the aglycone (**45**) of sativanone (Figure 7). The molecular formula of peaks **22**, **28**, **33**, and **41** (Figure 1F) differed from the corresponding ones of sativanone in that of an extra oxygen and the lack of the two hydrogens which could be a consequence of a methylenedioxy substitution instead of a methoxy group. The loss of H_2O and CO units could be observed on the MS/MS spectra at *m/z* 297.0753 ($C_{17}H_{13}O_5$) and 287.0909 ($C_{16}H_{15}O_5$). Proving that the substitution patterns of the A-ring of sativanone and the aglycone of these molecules are identical, the same ions at *m/z* 163.0388 and 135.0440 could be detected. Moreover, as an evidence of the methylenedioxy substituent, ions at *m/z* 257.0804 ($C_{15}H_{13}O_4$) [M + H-CO-CH_2O]$^+$ and 229.0857 ($C_{14}H_{13}O_3$) [M + H-2CO-CH_2O]$^+$ could be detected (Table 1). Regarding these aspects, the structures were putatively identified as the 7-*O*-glucoside (**22**), 4″ and 6″-*O*-glucoside malonates (**28** and **33**), and aglycone (**41**) of onogenin (Figure 7).

3.6 | Identification of the rare 2′-methoxy isoflavonoids

All the former discussed isoflavonoids, dihydroisoflavonoids, and pterocarpans were already known for *O. spinosa*[10]; however, peak **43** showed a *m/z* value for the aglycone which was not mentioned in *Ononis* species before. The formula calculated from the HR-MS data was $C_{17}H_{14}O_5$ (Table 1), which could refer to known compounds afrormosin, cladrin, or 2′-methoxy formononetin. As a result of CID, ions at *m/z* 284.0658 ($C_{16}H_{12}O_5$), 267.0649 ($C_{16}H_{11}O_4$), and 252.0412 ($C_{15}H_8O_4$) were detected, which could refer to structures [M + H-$CH_3^{\bullet}$]$^+$, [M + H-CH_3OH]$^+$, and [M + H-$CH_3^{\bullet}$-CH_3OH]$^+$, respectively. These fragments clearly showed that the molecule contained two methoxy groups. The product ion at *m/z* 243.1014 ($C_{15}H_{15}O_3$) is a result of the ejection of two CO molecules, which is diagnostic to isoflavonoid molecules.[12] The cleavage of the bond between the C-ring and B-ring could provide ions at *m/z* 163.0387 ($C_9H_7O_3$) [M + H-B-ring]$^+$ and 137.0596 ($C_8H_9O_2$) [B-ring+H]$^+$. Regarding this fragmentation pattern, the A-ring can only bear a hydroxy group, and the two methoxy groups are localized on the B-ring. Further evidence for the position of the substituents is provided by the fragment at *m/z* 148.0517 ($C_9H_8O_2$), which was presumed to be the rDA product $[^{1,3}B\text{-}CH_3^{\bullet}]^+$. This fragment corresponds to the $[^{1,3}B\text{-}CH_3^{\bullet}]^+$ ion in the MS/MS spectra of formononetin, and the mass difference is caused by an extra methoxy substituent. Since afrormosin has a methoxy and a hydroxy group on the A-ring and a single methoxy substituent on the B-ring, it could be excluded from the list of possible structures (Figure 5). Comparing the MS/MS spectra of cladrin[26,27] and 2′-methoxy formononetin[28] with our data, the peaks at *m/z* 163.0, 148.1, and 137.1 could be observed in all cases indicating the double methoxy substitution of the B-ring; however,

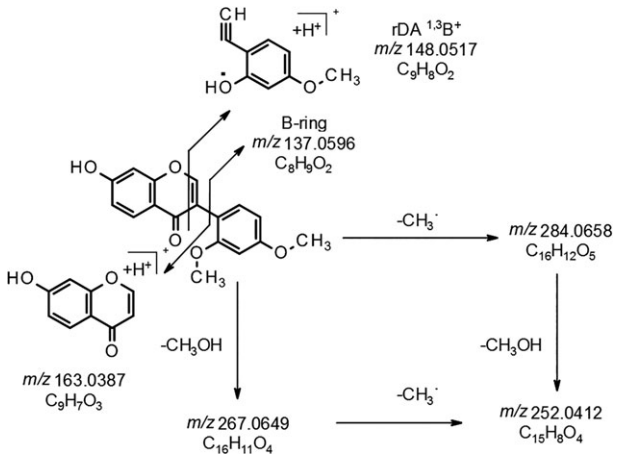


FIGURE 5 Proposed fragmentation pathway of 2′-methoxy formononetin

inspecting fragments in higher *m/z* regions, our detected peaks were clearly in agreement with the ones of the 2′-methoxy formononetin. 2′-Methoxy formononetin has been isolated from *Dalbergia parviflora* L.[29] and from *Eschscholtzia californica* L. (California poppy),[30] but not from *Ononis* species. The protonated formula calculated based on the exact mass for peak **40** ($C_{17}H_{13}O_6$) (Table 1) suggests that this compound contains an extra oxygen atom and misses two hydrogens atoms compared with 2′-methoxy formononetin. The origin of this difference could be the methoxy-methylenedioxy substitutions of isoflavonoids, just like in the case of formononetin-pseudobaptigenin, sativanone-onogenin, and medicarpin-maackiain pairs. The mass difference could be followed through the ions with smaller *m/z* ratio at 151.0388 ($C_8H_7O_3$) and 162.0310 ($C_9H_6O_3$), which are corresponding to the ones of 2′-methoxy formononetin at *m/z* 137.0596 ($C_8H_9O_2$) and 148.0517 ($C_9H_8O_2$) and were assigned as $[^{1,3}B\text{-}CH_3^{\bullet}]^+$ and $[B\text{-ring}+H]^+$ (Figure 6). Consequently, it can be deduced that the mass difference arose from the substitution pattern of the B-ring, meaning

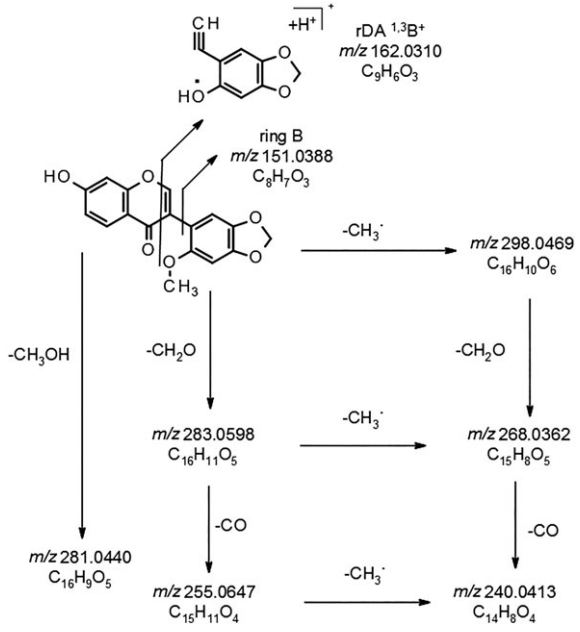


FIGURE 6 Proposed fragmentation pathway of cuneatin

that beside a methoxy group an additional methylenedioxy group is localized on it. As a proof of the mutual presence of OCH_3 and OCH_2O substituents, the loss of $CH_3^{\bullet}$, CH_3OH, and CH_2O units and their combination could be observed on the MS/MS spectrum at *m/z* 298.0469 ($C_{16}H_{10}O_6$) $[M + H\text{-}CH_3^{\bullet}]^+$, 283.0598 ($C_{16}H_{11}O_5$) $[M + H\text{-}CH_2O]^+$, 281.0440 ($C_{16}H_9O_5$) $[M + H\text{-}CH_3OH]^+$, and 268.0362 ($C_{15}H_8O_5$) $[M + H\text{-}CH_3^{\bullet}\text{-}CH_2O]^+$. Similarly to other isoflavones, the loss of CO units was also detectable at *m/z* 255.0647 ($C_{15}H_{11}O_4$) $[M + H\text{-}CH_2O\text{-}CO]^+$, 240.0413 ($C_{14}H_8O_4$) $[M + H\text{-}CH_3^{\bullet}\text{-}CH_2O\text{-}CO]^+$, and 212.0465 ($C_{13}H_8O_3$) $[M + H\text{-}CH_3^{\bullet}\text{-}CH_2O\text{-}2CO]^+$ (Table 1). In the literature, only cuneatin fulfills these structural criteria. Cuneatin was described in aerial parts of *Millettia oblata ssp. teitensis*,[31] aerial parts of *Retama sphaerocarpa*,[32] stem bark of *Dalbergia frutescens*,[33] *Eysenhardtia polystachya*,[34] aerial parts and roots of *Tephrosia maxima*[35] and most importantly, in *Cicer* species.[36] In his other work,[37] Ingham had shown the chemotaxonomic similarity of genus *Ononis* and *Cicer*, and the presence of cuneatin in both genus corroborates this idea.

Other confirmations of the presence of 2′-methoxy formononetin and cuneatin could be acquired from the biosynthesis of isoflavonoids. The final products of the isoflavonoid biosynthesis are medicarpin and maackiain (Figure 7). These phytoalexins with pterocarpan skeleton possess a methoxy and a methylenedioxy group, respectively. Formononetin serves as the parent compound of medicarpin, and through the calycosin-pseudobaptigenin route it could also be considered as the precursor of maackiain. Both formononetin and pseudobaptigenin undergo hydroxylation, converting to 2′-hydroxy formononetin and 2′-hydroxy pseudobaptigenin. These molecules were not detected in our sample; however, their 2′-*O*-methylated derivatives, 2′-methoxy formononetin, and cuneatin were confirmed. In the following step, the 2′-hydroxy isoflavones are reduced to the dihydro-derivatives: vestitone and sophorol.[38] Their 2′-*O*-methylated derivatives, sativanone and onogenin, are representative compound in *Ononis* species, as well as the pterocarpan medicarpin and maackiain.[39]

3.7 | Identification of beta amino acid derivatives

The most intense peaks in the chromatogram in positive ionization mode were **12**, **13**, **14**, **17**, **20**, and **23** (Figure 1). Based on our previous work,[11] these compounds were identified as homopipecolic acid esters of isoflavonoid glucosides. These molecules are analogous structures to glucoside malonates, but instead of a malonic acid, the beta amino acid homopipecolic acid is involved in the esterification. However, their fragmentation differs rather from the glucoside malonates, where solely the $^0Y^+$ ion and its fragments emerge and the glucose moiety along with the malonic acid cleave as a neutral fragment (Figure 4D). In the case of homopipecolic esters, the beta amino acid protonates relatively easily due to the secondary amine function enabling the detection of the fragments containing the glycosidic part as well (Table 1). These nitrogen-containing product ions were common for all six isoflavonoid derivatives, whereas the exact mass of the $^0Y^+$ ion and its smaller fractions promoted the identification of the aglycone. The whole homopipecolic acid isoflavonoid

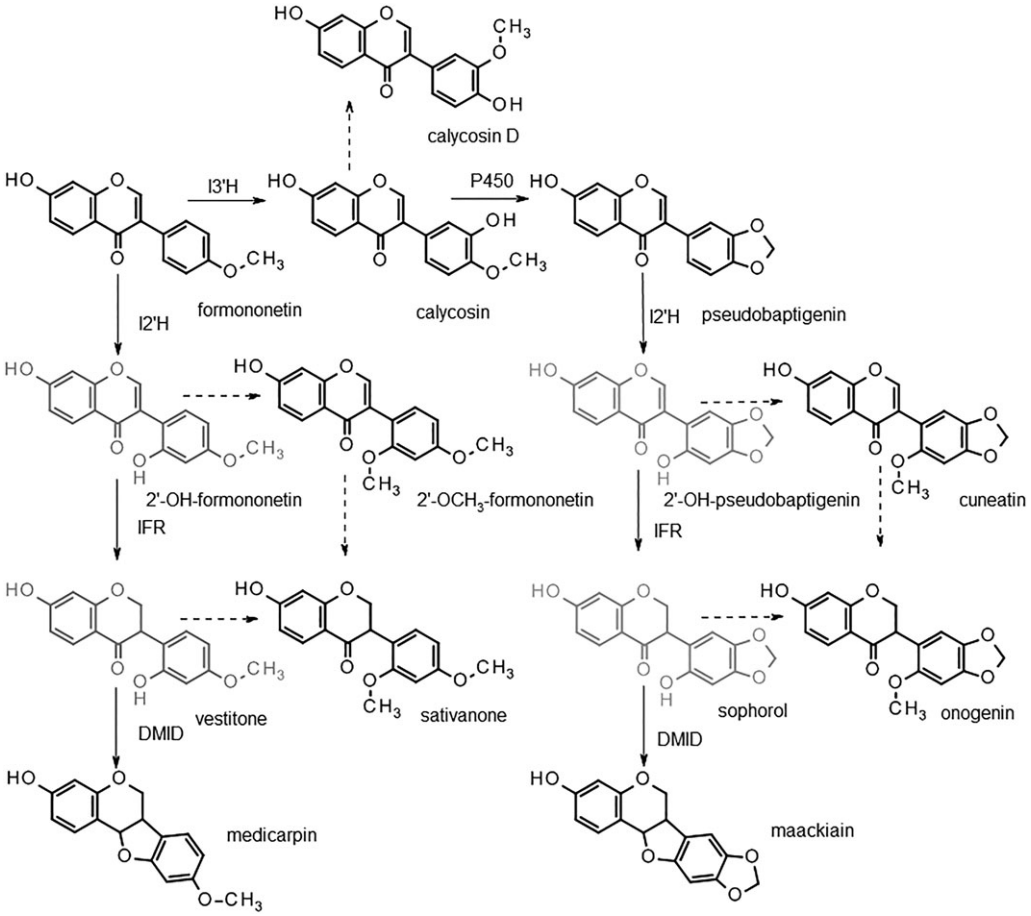


FIGURE 7 Biosynthesis of isoflavonoid and pterocarpans (based on the work of Davies and Schwinn, 2006). Grey: Compounds not detected. Dashed arrows: Unknown enzymatic steps

glucoside ester could cleave alongside the glycosidic bond, resulting in the intact protonated aglycone or the homopipecolic glucoside ester with a truncated glucose moiety at *m/z* 288.1435 ($C_{13}H_{22}NO_6$). As a protonated fragment at *m/z* 144.1017 ($C_7H_{14}NO_2$), the beta amino acid unit dissociates from the molecule. The cleavage of the heterocyclic ring solely could provide the ion at *m/z* 84.0814 ($C_5H_{10}N$) (Figure 8A). Interestingly, an analogous fragmentation pattern could be observed for compounds **3**, **5**, **8**, **9**, **10**, and **15**. The HR-MS/MS data suggested that these compounds contained the same isoflavonoid aglycones mentioned before; however, the complete formula is short of a CH_2 unit (Table 1). Investigating the MS/MS spectra, fragment ions at *m/z* 274.1284 ($C_{12}H_{20}NO_6$), 130.0862 ($C_6H_{12}NO_2$), and 70.0658 (C_4H_8N) were detected, indicating that the CH_2 difference arose from the heterocyclic ring (Figure 8B). As a homologue of homopipecolic acid, the pyrrolidine ring containing beta amino acid homoproline was presumed.

Although, these nitrogen-containing esters ionize extremely well in positive mode, their actual quantity compared with other isoflavonoid derivatives is quite low. The homoproline derivatives are orders of magnitude lower compared with the homopipecolic esters; therefore, their isolation for NMR experiments was not feasible. In order to verify that the compounds **3**, **5**, **8**, **9**, **10**, and **15** are homoproline derivatives, the sample was subjected to hydrolysis to free beta amino acids. This hydrolyzed sample was then spiked with the a standard solution of homopipecolic acid and homoproline in

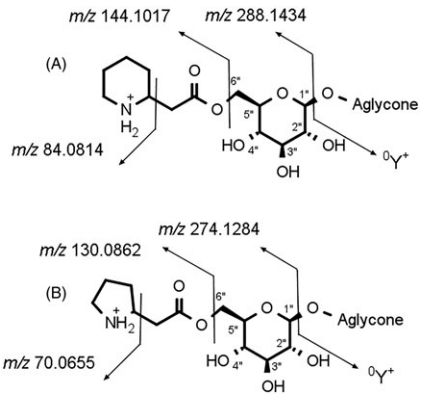


FIGURE 8 The proposed fragmentation pathways of homopipecolic acid (A) and homoproline esters (B)

aqueous medium, as they tend to form esters with methanol.[40] The original and the spiked samples were investigated by HPLC-MS/MS. As no differences were observed in retention time and in fragmentation pattern (Figure 9), it could be concluded that the samples actually contained the esters of homopipecolic acid and homoproline. Homopipecolic acid was described before in *Lycopodium* species as an intermediate of the synthesis of *Lycopodium* alkaloids,[41,42] while homoproline was isolated from *Asteraceae* species, as a precursor of pyrrolizidines,[40] but their co-occurrence has never been reported in plants and their biosynthetic origin is not known.

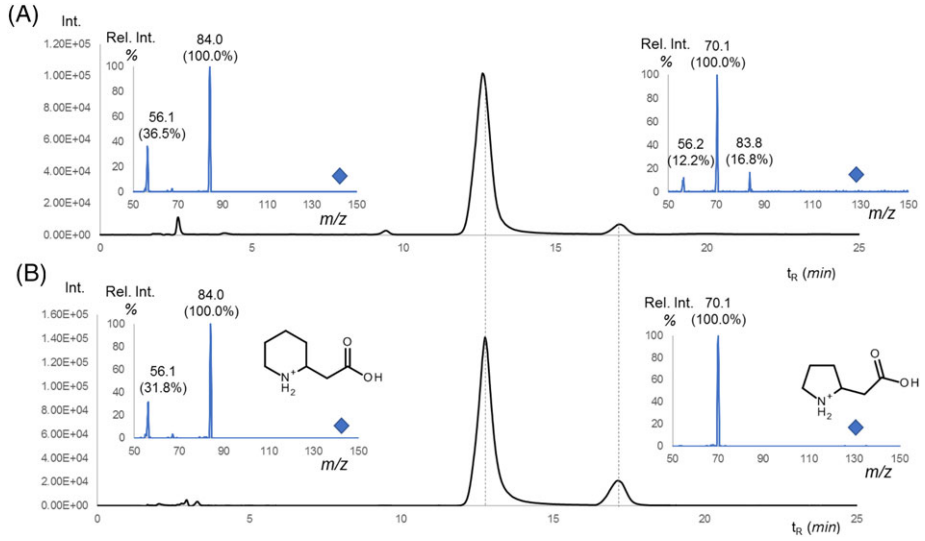


FIGURE 9 Chromatographic separation of homopipecolic acid (12.8 min) and homoproline (17.2 min) along with their product ion spectra in hydrolyzed plant sample (A) and hydrolyzed plant sample spiked with homopipecolic acid and homoproline standards (B)

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