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Determination of Saponins in Aerial Parts of Barrel Medic (*Medicago truncatula*) by Liquid Chromatography–Electrospray Ionization/Mass Spectrometry

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Triterpene saponins from aerial parts of *Medicago truncatula* cv. Jemalong A-17, *M. truncatula* Gaertn. var. *longispina* Urb., and *M. truncatula* Gaertn. var. *truncatula* were profiled and quantified using reverse-phase liquid chromatography with on-line photodiode array detection and electrospray ionization mass spectrometry (LC-PDA/ESI/MS/MS). The determination was based on standard curves obtained for the 18 available saponin standards, previously isolated from Jemalong A-17. Aerial parts of all three subspecies contained 17 saponins previously identified and also a substantial amount of astragaloside VIII (3-GlcA-Xyl-Rha soyasapogenol B), not previously reported in *M. truncatula*. The compositions of saponin mixtures were very similar in the three subspecies with three dominant groups, recognized as zanhic acid, medicagenic acid, and soyasapogenol glycosides. Relative proportions of these three groups were also similar in the three subspecies: var. *longispina* had 49.5, 48.1, and 2.4%; var. *truncatula*, 41.5, 53.4, and 5.1%; and Jemalong A-17, 42.1, 56.6, and 1.3% of zanhic acid, medicagenic acid, and soyasapogenol glycosides, respectively. Jemalong A-17 had 30% lower total content of saponins as compared to *M. truncatula* var. *longispina* and *M. truncatula* var. *truncatula*; in relation to the dry matter, var. *longispina* contained 0.22%, var. *truncatula*, 0.22%, and Jemalong A-17, 0.15% dry matter of saponins. If one takes into consideration that this determination was performed on spring-collected samples, it can be concluded that the concentration of saponins in *M. truncatula* is similar to the concentration in alfalfa (*Medicago sativa*); the proportions of the three groups of saponins in these species are slightly different from those found in alfalfa, having a higher content of zanhic acid glycosides.

KEYWORDS: Triterpene saponins; barrel medic; *Medicago truncatula*; liquid chromatography–mass spectrometry (LC-PAD/ESI/MS/MS)

INTRODUCTION

Over the past few years *Medicago truncatula* has emerged as a model legume for the study of plant functional genomics because of its short regeneration time, diploid character, and small genome (*1*). This species is a convenient model for studying various plant processes associated with both pathogenic and symbiotic interactions with microorganisms (*2*, *3*) and the unique secondary metabolism of legumes (*4*, *5*). Two classes of secondary metabolites are of interest in *M. truncatula*, flavonoids and triterpene saponins. Flavonoids (*5*–*7*) and triterpene saponins (*8*–*11*) are of interest due to a wide range of biological activities. Previous investigations on saponins isolated from *Medicago sativa* have shown that, depending on the structure, they possess antimicrobial activity, mainly against plant pathogens (*12*) and some yeasts pathogenic to humans (*13*), and anti-insect activity (*14*, *15*). They can also be toxic to monogastric animals and may reduce the digestibility of protein in ruminants (*16*). Some saponins exhibit pharmacological properties including anticholesterolemic, anticancer, and hemolytic activities (*17*).

Mass spectrometry has played an important role in phytochemistry. Prior to the introduction of electrospray ionization (ESI), fast atom bombardment mass spectrometry (FAB-MS) was employed to determine saponins. Recent studies have demonstrated the advantages of the use of multistage mass spectrometry for the characterization of constituents in crude extracts. Among various methods that have been applied to the analysis and identification of saponins, LC-MS appears to be most favorable. Two papers have been already published on the analysis of saponins in barrel medic using LC-MS (*9*, *11*). However, both were based on a limited number of saponin standards available, and full characterization and quatification of saponins were not possible.

In the present study we have identified and determined saponins in aerial parts of barrel medic using LC-ESI/MS/MS

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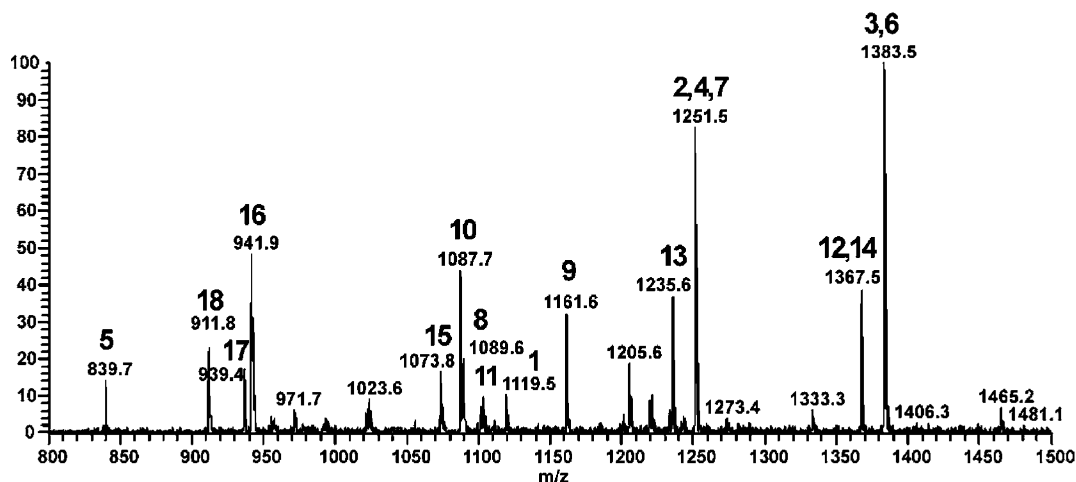


Figure 1. Direct injection ESI/MS of the mixture of *M. truncatula* saponins purified by solid-phase extraction.

based on the standards that were previously isolated and characterized by a combination of chromatographic and spectroscopic methods (*10*).

MATERIALS AND METHODS

Plant Material. Seeds of *M. truncatula* Jemalong A-17 were obtained from Dr. XianZhi He, The Samuel Roberts Noble Foundation, Ardmore, OK, where a voucher specimen is deposited, and from Dr. J. M. Prosperi, INRA-SGAP, Monpellier, France. Seeds of *M. truncatula* Gaertn. var. *longispina* Urb. (specimen MED 98/96) and *M. truncatula* Gaertn. var. *truncatula* (specimen MED 161/95) were obtained from Genebank, Zentralinstitut für Pflanzengenetic und Kulturpflanzenforschung, Gatersleben, Germany. Plants were cultivated in an experimental field of the Institute of Soil Science and Plant Cultivation in Pulawy, Poland. Plants were harvested at the beginning of flowering. The plant material was freeze-dried, finely powdered, and used for extraction.

Extraction. One gram of dried and finely powdered barrel medic tops (leaves and stems) was extracted overnight with 50 mL of 80% MeOH at room temperature. The extract was filtered, and the residues were additionally extracted twice by refluxing with 50 mL of 80% MeOH for 1 h. The extracts were combined, and the solvent was removed under reduced pressure.

Purfication. The crude extract was suspended in water and passed through a C_{18} Sep-Pak cartridge (Waters Associates) preconditioned with water. The cartridge was washed first with water to remove sugars and then with 40% MeOH to elute phenolics. Saponins were eluted with 80% MeOH, evaporated, and redissolved in MeOH (1 mL) for direct injection MS analyses. For HPLC 150 μL was transferred to another vial, and the solution was made up to a final volume of 1.5 mL with MeOH.

High-Performance Liquid Chromatography. An LC system consisting of a Finnigan Surveyor pump equipped with a gradient controller, an automatic sample injector, and a PDA detector was used. The separation was performed on a 250 × 4 mm i.d., 5 μm, Eurospher 100 C_{18} column (Knauer, Germany). A mobile phase consisted of 0.05% acetic acid in water (B), and 0.05% acetic acid in acetonitrile (A) was used for the separation. The flow rate was kept constant at 0.5 mL/min for a total run time of 90 min. The system was run with the following gradient program: from 18% A to 36% A in 55 min, from 36% A to 100% A in 20 min. The sample injection volume was 25 μL.

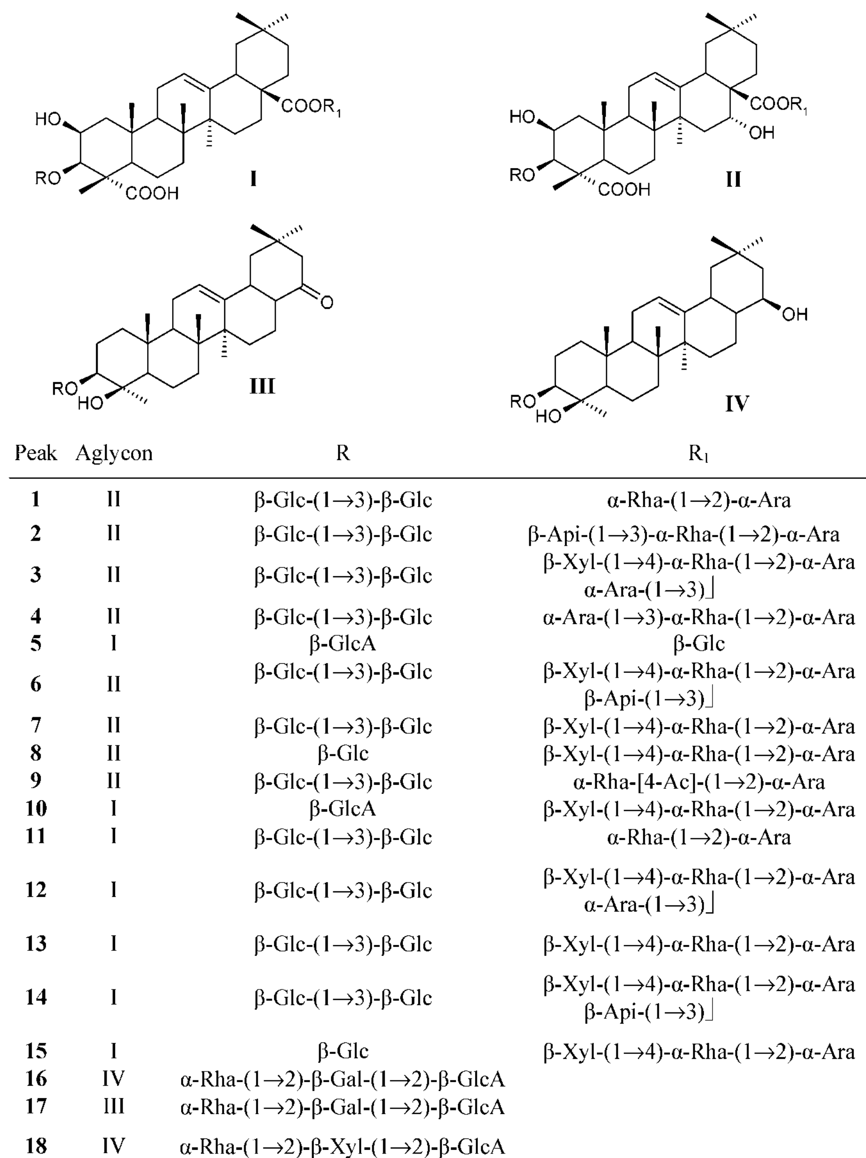
Mass Spectrometry. A Thermo Finnigan LCQ Adventage Max ion-trap mass spectrometer with an electrospray ion source was coupled to the HPLC system described above. The samples were introduced on column via an automatic sampler injector or direct injection by a syringe pump at a flow rate of 5 μL/min. The spray voltage was set to 4.2 kV and the capillary offset voltage to −60 V. All spectra were acquired at a capillary temperature of 220 °C. The calibration of the mass range (400−2000 Da) was performed in negative ion mode. Nitrogen was used as sheath gas, and the flow rate was 0.9 L/min. The maximum ion injection time was set to 200 ms.

Identification, Standards Preparation, and Quantitative Analysis. Three independent chromatographic runs were performed for each extract, and saponins were identified through comparison with authentic standards. These included retention times, cochromatography, and mass spectra. Quantitation was based on external standardization by employing calibration curves in the range of 0.0362−6.94 μg/mL. Standards were prepared in methanol prior to analysis. Quantitative analyses were based on the peak area calculated from selected ion chromatograms of the corresponding $[M - H]^-$ ion. Absolute quantifications were performed for 18 saponins. Microsoft Excel 2000 was used for the statistical analysis.

RESULTS AND DISCUSSION

Solid-phase extraction of *M. truncatula* aerial part saponins on C-18 Sep-Pak cartridges provided samples with the matrix that did not show many impurities interfering when injected by the syringe pump to the ESI-MS. The direct injection negative ion mode MS spectrum of such extracts showed the presence of a number of peaks in mass range of 800−1500 amu (**Figure 1**). Thirteen of these peaks could be easily ascribed to saponins that were previously separated and identified in *M. truncatula* aerial parts (*10*). Three peaks, however, could be ascribed to mixtures of saponins as the three compounds (**2**, **4**, and **7**) had identical masses of 1252, two saponins (**12** and **14**) had masses of 1368, and compounds **3** and **6** had masses of 1384. Thus, on the basis of direct injection analyses it was not possible to recognize all of the compounds present in the matrix. However, it was possible to confirm our previous finding on the occurrence of soyasaponin I [3-(Rha-Gal-GlcA) soyasapogenol B] and 3-(Rha-Gal-GlcA) soyasapogenol E glycosides for which quasi-molecular ions were observed at 941 and 939 amu, respectively. Additionally, there was one extra quasi-molecular ion observed at 911 amu, which was by 30 amu different from soyasaponin I and was temporarily identified as astragaloside VIII [3-(Rha-Xyl-GclA) soyasapogenol B]. Its identity was confirmed further by comparing MS/MS degradation patterns after HPLC separation with MS obtained for the authentic standard (*18*), and the compound was included in the standard mixture. The structures of identified saponins are presented in **Figure 2**.

Once the major components of *M. truncatula* saponins were known, the mixture of their standards was prepared to optimize chromatographic conditions. Due to the fact that a number of zanhic acid glycosides showed similarity in structures resulting in nearly identical polarities, optimization of separation conditions was essential for successful separation. After a number of



Peak	Aglycon	R	R1
1	II	β-Glc-(1→3)-β-Glc	α-Rha-(1→2)-α-Ara
2	II	β-Glc-(1→3)-β-Glc	β-Api-(1→3)-α-Rha-(1→2)-α-Ara
3	II	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara α-Ara-(1→3)⌋
4	II	β-Glc-(1→3)-β-Glc	α-Ara-(1→3)-α-Rha-(1→2)-α-Ara
5	I	β-GlcA	β-Glc
6	II	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara β-Api-(1→3)⌋
7	II	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
8	II	β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
9	II	β-Glc-(1→3)-β-Glc	α-Rha-[4-Ac]-(1→2)-α-Ara
10	I	β-GlcA	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
11	I	β-Glc-(1→3)-β-Glc	α-Rha-(1→2)-α-Ara
12	I	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara α-Ara-(1→3)⌋
13	I	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
14	I	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara β-Api-(1→3)⌋
15	I	β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
16	IV	α-Rha-(1→2)-β-Gal-(1→2)-β-GlcA	
17	III	α-Rha-(1→2)-β-Gal-(1→2)-β-GlcA	
18	IV	α-Rha-(1→2)-β-Xyl-(1→2)-β-GlcA	

Figure 2. Chemical structures of saponins used as a standards for LC-MS/MS determination: **I**, medicagenic acid; **II**, zanhic acid; **III**, soyasapogenol E; **IV**, soyasapogenol B.

trials, the best separation was obtained with a gradient changing very slowly from 18 to 36% of solution A (0.05% acetic acid in acetonitrile) over 55 min followed by quick change from 36 to 100% A over 20 min. This gradient allowed the best separation of medicagenic glycosides and zanhic acid glycosides in the first part of analysis (t_R between 25 and 45 min). To elute soyasapogenol B and E glycosides, a higher concentration of acetonitrile was needed, and after the gradient was accelerated, they eluted with t_R around 65 min (**Figure 3B**).

Full identification of the saponins present in the standard mixture was achieved first by comparing their mass spectrometric data and then by cochromatography with the authentic standards. Quantitative changes of the peak areas in spiked chromatograms, calculated from selected ion chromatograms of corresponding $[M - H]^-$ ions, were also a very valuable means of positioning peaks. This was especially helpful for recognition of zanhic acid glycosides **2**, **4**, and **7**, which had the same pseudomolecular ion $[M - H]^-$ at m/z 1251 but under experimental conditions separated satisfactorily enough to be quantified (**Figure 3C**). A similar situation was found for another two zanhic acid bidesmosides **3** and **6** (**Figure 3D**). They also had sugar substitutions differing only in one terminal pentose, which was arabinose in compound **3** and apiose in saponin **6**. Separation was, however, not successful in the case of medicagenic acid glycosides **12** and **14**, having identical masses of 1368 amu and sugar chain composition identical to those of zanhic acid glycosides **3** and **6**, respectively. Saponins **12** and **14** under all experimental conditions gave one symmetrical peak with t_R of 36.87 min (**Figure 3E**). The retention times, mass values, and CID fragments of all tested standards are shown in **Table 1**. Under these separation conditions saponin mixtures extracted from plant material and purified by SPE were also successfully separated for quantification (**Figure 3A**).

For the determination of individual saponins it was necessary to prepare standard calibration curves. To this purpose, standards were separated in a series of concentrations ranging between 0.0362 and 6.944 μg/mL. In this range of concentrations the standards curves showed good linearity, with R^2 values of 0.99 (in some exceptions this was lower, but never below 0.90). They were used for quantification of individual compounds in *M. truncatula* subspecies. For evaluation of the instrument precision and extraction/purification repeatability, six samples from the same plant powder were independently extracted and purified with the SPE procedure. For each sample three independent LC-

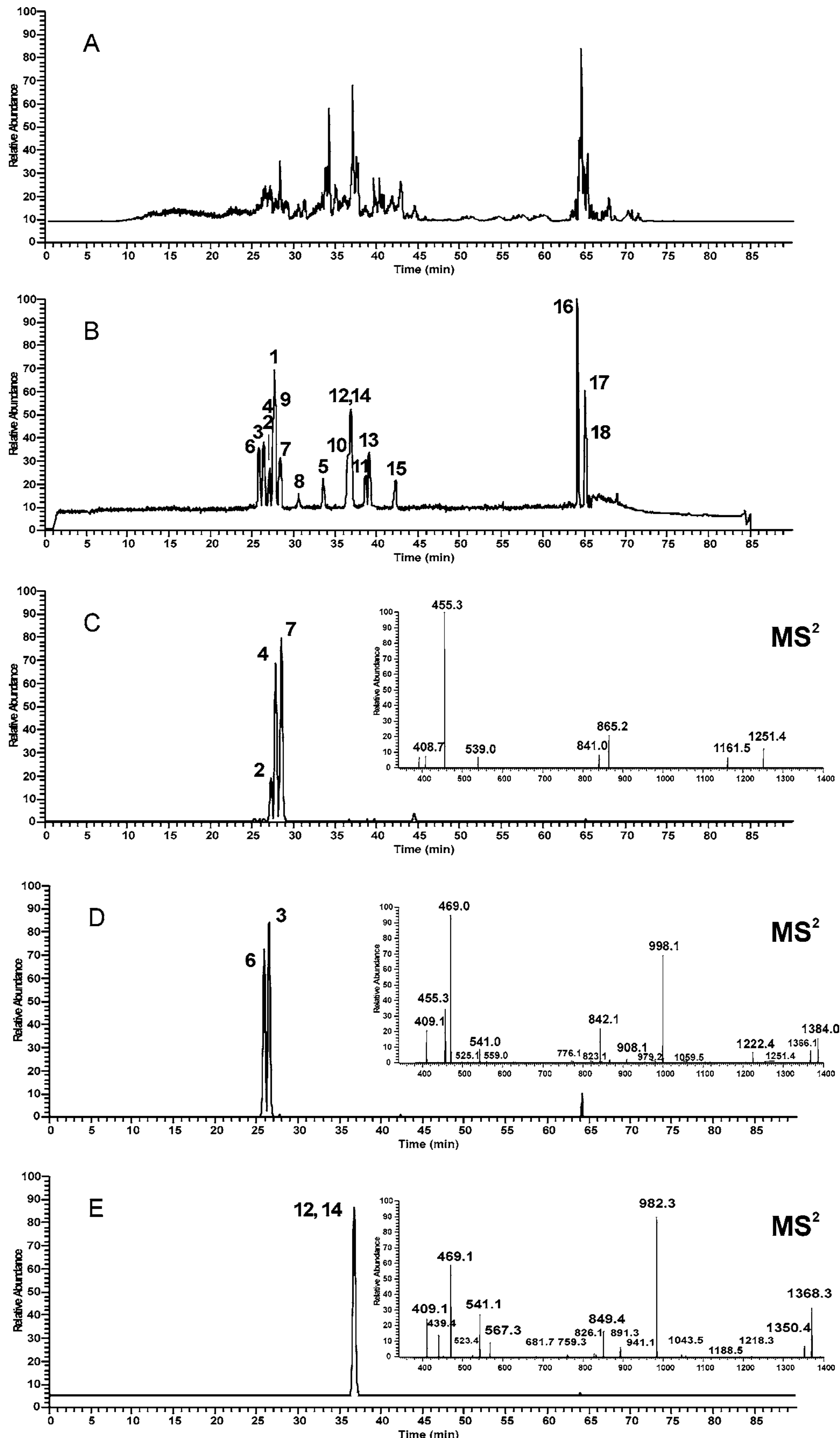


Figure 3. Total and selected ion chromatograms of *M. truncatula* cv. Jemalong A-17, aerial part saponins: (**A**) total ion chromatogram obtained by negative-ion LC-ESI/MS of SPE purified plant extract; (**B**) total ion chromatogram obtained by negative-ion LC-ESI/MS of mixture of 18 standard saponins; (**C**) selective ion chromatogram (*m*/*z* 1257 $[M-H]^-$) representing saponins **2**, **4**, and **7**; (**D**) selective ion chromatogram (*m*/*z* 1383 $[M-H]^-$) representing saponins **3** and **6**; (**E**) selective ion chromatogram (*m*/*z* 1367 $[M-H]^-$) representing saponins **12** and **14**. Peak areas from the selective ion chromatograms were used to calculate absolute concentrations based on the standard response curves of 18 *M. truncatula* aerial part saponin standards. MS/MS spectra were obtained by direct introduction ESI/MS/MS.

Table 1. Saponins Observed by Negative Ion LC-ESI/MS in *M. truncatula*

Peak No	Saponin	Rt (min)	ESI/MS	ESI/MS/MS
6	3-Glc-Glc-28- Ara-Rha-Xyl Api ┘ zanhic acid	25.85	1383.80[M-H]$^-$	998.10[M-H-2 Glc-COOH-H_2O]$^-$, 842.20[M-H-Api-Xyl-Ara-Rha]$^-$, 455.30[M-H-2 Glc- Api-Xyl-Ara -Rha-COOH-H_2O]$^-$
3	3-Glc-Glc-28-Ara-Rha-Xyl Ara ┘ zanhic acid	26.43	1383.90[M-H]$^-$	998.20[M-H-2 Glc-COOH-H_2O]$^-$, 841.20[M-H-Ara-Xyl-Ara-Rha]$^-$, 455.30[M-H-2 Glc- Ara-Xyl-Ara -Rha-COOH-H_2O]$^-$
2	3-Glc-Glc-28-Ara-Rha-Api zanhic acid	27.11	1251.60[M-H]$^-$	865.20[M-H-2 Glc-COOH-H_2O]$^-$, 841[M-H-Api-Ara-Rha]$^-$, 455.20[M-H-2 Glc-Api-Ara-Rha-COOH-H_2O]$^-$
4	3-Glc-Glc-28-Ara-Rha-Ara zanhic acid	27.57	1251.90[M-H]$^-$	865.20[M-H-2 Glc-COOH-H_2O]$^-$, 842.10[M-H-Ara-Ara-Rha]$^-$, 455.40[M-H-2 Glc-Ara-Ara-Rha-COOH-H_2O]$^-$
1	3-Glc-Glc-28-Ara-Rha zanhic acid	27.67	1119.10[M-H]$^-$	841.40[M-H-Ara-Rha]$^-$, 733.13[M-H-2 Glc-COOH-H_2O]$^-$, 455.27[M-H-2Glc-Ara-Rha-COOH-H_2O]$^-$
9	3-Glc-Glc-28-Ara-Rha-4-Ac zanhic acid	27.79	1161.70[M-H]$^-$	840.70[M-H-Rha-Ara-COMe]$^-$, 775[M-H-2 Glc-COOH-H_2O]$^-$, 733.10[M-H-2 Glc-COMe-COOH-H_2O]$^-$, 455.20[M-H-2 Glc-Rha-Ara-COMe-COOH-H_2O]$^-$
7	3-Glc-Glc-28-Ara-Rha-Xyl zanhic acid	28.41	1251.80[M-H]$^-$	866.20[M-H-2 Glc-COOH-H_2O]$^-$, 842[M-H-2 Pen-Rha]$^-$, 455.30[M-H-2 Glc-2 Pen-Rha-COOH-H_2O]$^-$
8	3-Glc-28-Ara-Rha-Xyl zanhic acid	30.65	1089.50[M-H]$^-$	865.40[M-H-Glc-COOH-H_2O]$^-$, 455.30[M-H-Glc-2 pentoses-Rha-COOH-H_2O]$^-$
5	3-GlcA-28-Glc medicagenic acid	33.59	839.50[M-H]$^-$	663.50[M-H-GlcA]$^-$, 502.20[M-H-GlcA-Glc]$^-$
10	3-GlcA-28-Ara-Rha-Xyl medicagenic acid	36.51	1087.80[M-H]$^-$	911.80[M-H-GlcA]$^-$, 678.50[M-H-Xyl-Ara-Rha]$^-$, 501.50[M-H-GlcA-Xyl-Ara-Rha]$^-$
12	3-Glc-Glc-28-Ara-Rha-Xyl Ara ┘ medicagenic acid	36.89	1367.10[M-H]$^-$	981.30[M-H-2 Glc-COOH-H_2O]$^-$, 849.20[M-H-2 Glc-pentose-COOH-H_2O]$^-$, 439.90[M-H-2 Glc-Ara-Xyl-Ara-Rha-COOH-H_2O]$^-$
14	3-Glc-Glc-28- Ara-Rha-Xyl Api ┘ medicagenic acid	36.89	1367.90[M-H]$^-$	982.30[M-H-2 Glc-COOH-H_2O]$^-$, 849.40[M-H-2 Glc-pentose-COOH-H_2O]$^-$, 439[M-H-2Glc-Api-Xyl-Ara-Rha-COOH-H_2O]$^-$
11	3-Glc-Glc-28-Ara-Rha medicagenic acid	38.72	1103.60[M-H]$^-$	717.10[M-H-2 Glc-COOH-H_2O]$^-$, 439.90[M-H-2 Glc-Ara-Rha-COOH-H_2O]$^-$
13	3-Glc-Glc-28-Ara-Rha-Xyl medicagenic acid	39.14	1235.90[M-H]$^-$	1055.60[M-H-Glc]$^-$, 912.30[M-H-2 Glc]$^-$, 850.30[M-H-2 Glc-COOH-H_2O]$^-$, 718.10[M-H-2 Glc-Xyl-COOH-H_2O]$^-$, 439.50[M-H-2 Glc-Xyl-Ara-Rha-COOH-H_2O]$^-$
15	3-Glc-28-Ara-Rha-Xyl medicagenic acid	42.28	1073.70[M-H]$^-$	892.90[M-H-Glc-H_2O]$^-$, 849.20[M-H-Glc-COOH-H_2O]$^-$, 662.80[M-H-Xyl-Ara-Rha]$^-$, 439.90[M-H-Glc-Xyl-Ara-Rha-COOH-H_2O]$^-$
16	3-Rha-Gal-GlcA soyasapogenol B (soyasaponin I)	64.15	941.60[M-H]$^-$	923.40[M-H-H_2O]$^-$, 879.30[M-H-COOH-H_2O]$^-$, 733.20[M-H-Rha-COOH-H_2O]$^-$, 571.40[M-H-Rha-Gal-COOH-H_2O]$^-$, 391.30[M-H-Rha-Gal-GlcA-COOH-H_2O]$^-$
17	3-Rha-Gal-GlcA soyasapogenol E	65.10	939.50[M-H]$^-$	921.90[M-H-H_2O]$^-$, 775.80[M-H-Rha-H_2O]$^-$, 613.90[M-H-Rha-Gal-H_2O]$^-$, 437.70[M-H-Rha-Gal-GlcA-H_2O]$^-$
18	3-Rha-Xyl-GlcA soyasapogenol B (astragaloside VIII)	65.42	911.8[M-H]$^-$	849.30[M-H-COOH-H_2O]$^-$, 703.40[M-H-Rha-COOH-H_2O]$^-$, 571.20[M-H-Rha-Xyl-COOH-H_2O]$^-$, 395.70[M-H-Rha-Xyl-GlcA-COOH-H_2O]$^-$

MS runs were performed. It was shown that for repetition of the same sample ($n = 3$) the relative standard deviation was ∼3% (with some exceptions where it was higher). However, in general, variation of the data caused by the instrument was satisfactory. Relative standard deviations between results obtained for six independent extractions ranged from 2.3 to 12%. Considering that it is difficult to obtain homogeneous plant material and that preparation of samples for the analysis included several steps of extraction/purification, this relative standard variation was satisfactory and comparable to other reported LC-MS data for *M. truncatula* (*11*) and soybean saponins (*19*).

The three *M. truncatula* subspecies differed in the total concentration of saponins (**Table 2**). Jemalong A-17 had a saponin content 30% lower as compared to *M. truncatula* var. *longispina* and *M. truncatula* var. *truncatula*. In relation to the dry matter var. *longispina* contained 0.22%, var. *truncatula*, 0.22%, and Jemalong A-17, 0.15% of saponins. Considering that plants were harvested in the spring (first cut) when saponin content is lowest over the whole season (*20*), this concentration was comparable to the amount that can be found in alfalfa with medium saponin content.

The compositions of the saponin mixtures were very similar in the three subspecies with three dominant groups recognized as zanhic acid, medicagenic acid, and soyasapogenol glycosides. Relative proportions of these three groups were also similar in the three subspecies: var. *longispina* had 49.5, 48.1, and 2.4%; var. *truncatula*, 41.5, 53.4, and 5.1%; and Jemalong A-17, 42.1, 56.6, and 1.3% of zanhic acid, medicagenic acid, and soyasapogenol glycosides, respectively. These data were slightly different from those reported in alfalfa (*M. sativa*) tops (*21*), where proportions of 20, 67, and 13% of total saponins were found for zanhic acid, medicagenic acid, and soyasapogenol glycosides, respectively. The concentration of soyasapogenol glycosides was marginal in *M. truncatula*. The other two groups, medicagenic and zanhic acid glycosides, occurred in nearly 1:1 ratio. This finding remains in disagreement with a previous study of Huhman an co-workers (*11*), who found that aerial parts contained only traces (0.6% of total) of zanhic acid glycosides and soyasapogenol glycosides made up 26%, whereas medicagenic acid glycosides were dominant with 73% of total. Also, 3-Glc-Glc-Glc-23-Ara-28-Ara-Rha-Xyl-zanhic acid, which was reported by Huhman et al. and identified previously in alfalfa

Table 2. Concentration of Individual Saponins and Total Saponins in Three *M. truncatula* Subspecies of Harvest I

compd	*M. truncatula* var. *longispina* (μg/g of dm)	*M. truncatula* var. *truncatula* (μg/g of dm)	*M. truncatula* Jemalong A 17 (μg/g of dm)
1	89.6	93.7	61.9
2	156.7	96.8	83.6
3	91.8	139.1	75.7
4	89	55.4	70.6
5	137	102.1	65.7
6	136.3	171.9	106.3
7	231.5	251.3	145.5
8	203	145.2	125.3
9	131.7	tr[a]	tr
10	354.3	279.8	287.2
11	303.1	502.7	259.4
12+14	52.5	72.1	57
13	97.4	103.5	72
15	103.1	94	99.8
16	42.6	66.3	20.7
17	6.8	40.2	tr
18	5.4	10.2	tr
total	2231.8	2224.3	1530.7

[a] Traces.

aerial tops (*22*), was not found in the present research to be a component of *M. truncatula*. Its zanhic acid glycosides differed from those reported in alfalfa both in the number of sugars (shorter chains substituted at the 3-*O* position in *M. truncatula*) and in the type of linkage (1→3) in *M. truncatula* and (1→2) in *M. sativa*. Zanhic acid glycosides of *M. truncatula* seem to form a more divergent group, and their concentrations were higher than in alfalfa. Their concentration in var. *longispina* was 0.11%; in var. *truncatula*, 0.09%; and in Jemalog A-17, 0.06% of dry matter, which was higher than in alfalfa collected at the same sampling date. Studies on zanhic acid glycoside concentrations in 10 varieties of alfalfa (*M. sativa*) grown for three consecutive years showed that these ranged between 0.02 and 0.06% of dry matter in the first cut (end of May) and increased to 0.08−1.15% in the second cut and to 0.15−0.3% in the middle of summer (*23*).

Structural and concentration similarities of the three subspecies of *M. truncatula* obtained in this research prove the correctness of previous findings on the isolation and identification of saponins (*10*). They indicate that all saponins isolated were the major components found in this plant. The present research showed that besides a number of zanhic acid glycosides, there are several medicagenic acid glycosides found in substantial amounts in *M. truncatula* aerial parts, which remains in disagreement with a previous finding (*11*) where 3-GlcA, 28-Ara-Rha-Xyl medicagenic acid was found to be the sole, dominant compound. Several factors could be responsible for these differences. The first and most important factor seems to be the lack of appropriate standards in the previous work (*11*) and dependence predominantly on interpretation of mass spectra. For proper metabolomic work it seems to be essential for any plant material to be first fully characterized phytochemically with classical methods to produce standards. As shown in this research discrimination between structurally different compounds having the same masses is not possible without the availability of appropriate standards. Also, the type of linkage between sugars is not possible solely from MS/MS interpretation. A second important factor, which should also be recognized, is the age and conditions of plant cultivation. Previous work (*11*) was performed on plants grown in a greenhouse, whereas the present data are based on field-grown plants cultivated in natural sunlight. How important this factor could be is difficult to assess without further research, but from our previous work on alfalfa seedlings (*24*) it was evident that saponin profiles in greenhouse seedlings and field-grown plants were nearly identical. However, the influence of age and cultivation conditions cannot be excluded. The present LC-PAD/ESI/MS method is based on the full spectrum of available saponin standards present in *M. truncatula* and gives a good basis for further research on this species.

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