JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantification of Saponins in Aerial and Subterranean Tissues of *Medicago truncatula*

DAVID V. HUHMAN,† MARK A. BERHOW,‡ AND LLOYD W. SUMNER*,†

Plant Biology Division, The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, Oklahoma 73401, and Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604

Triterpene saponins from aerial and subterranean organs of *Medicago truncatula* cv. Jemalong A-17 were qualitatively profiled and quantified using reverse-phase HPLC with on-line photodiode array detection and electrospray-ionization mass spectrometry (HPLC/PDA/ESI/MS). Absolute quantifications were performed for 3-*O*-*β*-D-glucopyranosyl-medicagenic acid and soyasaponin 1 (3-*O*-[α-L-rhamnopyranosyl(1→2)-*β*-D-galactopyranosyl(1→2)-*β*-D-glucuronopyranoside] soyasapogenol B), whereas relative quantifications were determined for 29 other saponins in root, stem, leaf, seedpod, and seed. Roots contained the greatest total amount of saponins followed by leaf and seed, respectively. The quantitative data also reveal the differential accumulation of triterpene saponins in the various organs of *M. truncatula.* Specifically, relatively higher quantities of medicagenic acid conjugates accumulated in leaf and seed, whereas relatively higher levels of soysapogenol conjugates were observed in root. The differential accumulation of specific triterpene saponins is suggestive of spatially differentiated biosynthesis and/or biological function.

KEYWORDS: Triterpene saponins; *Medicago truncatula*; barrel medic; quantification; high-performance liquid chromatography−mass spectrometry (HPLC/MS)

INTRODUCTION

Saponins are triterpenoid or steroidal glycosides that occur in a large number of plant species (*1*). These species include primarily dicots, but also selected monocots (*2*). The sapogenin aglycons are often substituted with a varying number of sugars via an ether and or ester bond at one or more glycosylation sites (*3*). Saponins are generally associated with plant defense but have also been associated with a wide range of other biological properties (*4*). These include deterrence to insects (*5*), antifungal properties (*6*, *7*), and poor digestibility in ruminants (*8*, *9*). Although traditionally viewed as antifeedants in forages, recent studies have reported beneficial anti-inflammatory (*10*, *11*), anticancer (*12*, *13*), and adjuvant (*14*, *15*) properties for saponins. Although these compounds have significant biological importance, very little is known about their distribution or biosynthesis in plants.

Metabolic profiling was recently used to assess and tentatively identify a large number of saponins in the model legume *Medicago truncatula* (*16*), and a functional genomics approach is being used to address the biosynthesis of these natural products in *M. truncatula* (*17*). This investigation provides additional quantitative information on the spatial accumulation of saponins in various *M. truncatula* organs. A better understanding of the qualitative, quantitative, and spatial accumulation of saponins is critically important to advancing our knowledge of how these compounds are related to specific resistance mechanisms and how best to approach metabolic engineering of these compounds.

Numerous methods have been previously reported for the quantification of saponins, and each offers its own caveats. Published methods include colorimetric reaction assays (*9*), which are both easy and sensitive but susceptible to interference from pigments or other components of the extract. Other less common methods include the use of ichthyotoxicity (*18*) as well as hemolytic methods, which are very structure dependent (*9*). The disadvantage of bioassays is their dependence upon the presence of specific biologically active saponins, which may not reflect an accurate measurement of the total saponin content of the plant. For example, it has been shown that medicagenic acid, zhanic acid, and hederagenin have higher biological activity than soyasapogenols (*19*), but all are present in alfalfa. Thus, these bioassays would be more representative of the acidic saponin rather than the soyasapogenol content.

Other more rigorous methods have been reported and include thin-layer chromatography (TLC), gas chromatography (GC), GC/mass spectrometry (GC/MS), high-performance liquid chromatography (HPLC), and HPLC/MS (*20*). Analysis and detection by GC/MS requires compound volatilization; therefore, many biological compounds require derivatization. For saponins, this is usually performed by hydrolysis of the saponin followed

* Corresponding author [telephone (580) 224-6710; fax (580) 224-6692; e-mail lwsumner@noble.org].
† The Samuel Roberts Noble Foundation.
‡ U.S. Department of Agriculture.

10.1021/jf0482663 CCC: $30.25
Published on Web 02/22/2005

by methylation and acetylation of the sapogenin (*5*). The hydrolyzed sugars are identified by further derivatization and GC/MS analysis or by TLC using appropriate standards. Separation and detection of saponins by HPLC/UV (*21*−*23*) is an alternative to GC/MS due to its ability to separate nonvolatile polar compounds without derivatization. These methods have been particularly useful for 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) conjugates, which have a strong absorbance at 292 nm (*23*). However, a large proportion of saponins lack a strong chromophore and, therefore, direct UV detection yields poor sensitivity for non-DDMP-conjugated saponins (*23*). Derivatization of saponins with 4-bromophenacyl bromide and 18-crown-6 can improve the UV detection sensitivity of saponins; however, this requires additional steps, and the derivatized compounds are reportedly unstable at room temperature after 48 h (*24*, *25*). More recently, LC/MS has been used to analyze saponin content in legumes and provides good sensitivity and selectivity for a wide range of saponins without the need for derivatization (*16*, *23*, *26*−*28*). In addition, sample enrichment techniques such as solid-phase extraction (SPE) (*29*) have been used to improve method sensitivity and 1 pmol limits of detection reported for soyasaponin 1 (*16*); however, these further steps introduce additional experimental variability. In our hands, experimental variability was observed to increase as much as 20% between samples when SPE was employed for *M. truncatula* extracts. Finally, identification and quantification of saponins are often performed through comparative analyses with authentic standards; however, this is complicated by the limited availability of authentic saponin standards from commercial sources.

Previously, 15 saponins in alfalfa (cultivars Apollo, Radius, and Kleszczewska) and 27 saponins in *M. truncatula* (cv. Jemalong) were separated and tentatively identified using HPLC coupled to negative-ion electrospray ionization (ESI) and mass selective detection ESI/HPLC/MS/MS (*16*). The above report shows that the root profiles of saponins in different cultivars of alfalfa were similar, but the root profile of *M. truncatula* was more complex. The primary goal of this study was to quantify individual saponins in various organs of *M. truncatula* to gain a better understanding of the spatial accumulation of different saponins. The secondary goal of this project was to improve the experimental method and to eliminate nonessential steps to allow greater throughput. To the authors' best knowledge, this is the first report on the quantification of saponins in *M. truncatula* root, leaves, stem, seedpod, and seeds.

MATERIALS AND METHODS

Biological Materials. *M. truncatula* (Jemalong, cv. A17) plants were grown in a highly controlled environment using a Percival E-54U growth chamber maintained at 90% humidity and at an average temperature of 24 °C day (16 h) and 20 °C night (8 h). Plants were grown in Turface MVP (Profile Products, Buffalo Grove, IL) medium using an illumination intensity of 216 μmol/s/m^2. Plant tissue/organs were harvested at 13 weeks of age, which corresponds to ~2.5 weeks after seed set.

Saponin Extraction. Differentiated aerial plant organs were dissected, immediately frozen in liquid nitrogen, and lyophilized to eliminate enzymatic activity. This procedure was repeated on the roots following removal from the Turface medium. Lyophilized tissues were powdered using a mortar and pestle. Then 10.00 ± 0.06 mg of tissue was weighed into a 1 dram (3.8 mL) glass vial and extracted with 0.5 mL of 80% methanol at room temperature for 2 h on an orbital shaker. HPLC grade methanol was obtained from J. T. Baker (Philipsburg, NJ). Samples were centrifuged at 2200*g* for 20 min at 4 °C, and 200 μL aliquots were transferred to HPLC autosample vials. Samples were analyzed by gradient elution, reverse-phase HPLC with simultaneous on-line UV and mass selective detection.

Table 1. Absolute Concentrations of 3-Glc-Medicagenic Acid and Soyasaponin 1 in Various *M. truncatula* Tissues Including Correlation Coefficients for Calibration Curves

tissue	3-Glc-medicagenic acid (ng/mg of dw)	SD[a]	R^2 [b]	soyasaponin 1 (ng/mg of dw)	SD	R^2 [b]
roots	257	47.7	0.9904	692	262	0.997
stem	0.141	0.051	0.9995	288	27.6	0.9974
leaf	ND[c]		0.9998	237	78.1	0.9981
seedpod	0.939	0.986	1.0	0.628	0.233	0.9965
seed	2.11	1.73	0.9998	60.8	27.7	0.9985

[a] SD, standard deviation of replicate analyses of eight independent plants. [b] R^2, coefficient of determination of the linear regression for each standard curve generated from authentic standards of 3-Glc-medicageinc acid or soyasaponin 1. [c] ND, none detected or below level of detection.

High-Performance Liquid Chromatography/Mass Spectrometry. Burdick and Jackson HPLC grade acetonitrile and water were obtained from VWR International. Double-distilled PPB/Teflon grade acetic acid was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). HPLC separation was achieved using a 250 × 4.6 mm i.d., 5 μm, reverse-phase, C18 column (J. T. Baker). Samples were eluted with a linear H_2O/acetonitrile gradient, 95:5 to 5:95, in 90 min, at a flow rate of 0.8 mL/min. The water was adjusted with acetic acid to a final concentration of 0.1%.

All mass spectra were acquired using a Bruker Esquire LC equipped with an electrospray ionization (ESI) source. Negative-ion ESI was performed using a source voltage of 3000 V and a capillary offset voltage of −70.7 V. Nebulization was achieved using nitrogen gas at a pressure of 70 psi. Desolvation was aided by the use of a nitrogen counter current gas at a pressure of 12 psi. The capillary temperature was set at 360 °C. Mass spectra were recorded over the range of *m*/*z* 50−2200. The Bruker ion trap was operated under an ion current control of 20000, maximum acquisition time of 100 ms, and trap drive setting of 60.

Qualitative Identification of Saponins, Standards Preparation, and Quantitative Analysis. Saponins were tentatively identified through the comparison with authentic standards using multiple and orthogonal dimensions of chemical characterization similar to that previously described (*16*). These include HPLC retention times, mass spectra, and tandem mass spectral data. Absolute and relative quantifications of saponins were performed using the standards 3-Glc-medicagenic acid and soyasaponin 1 (3-Rha-Gal-GlcA-soyasapogenol B). 3-Glc-medicagenic acid was purified from alfalfa (*24*, *30*) and kindly provided by W. Oleszek, Institute of Soil Science and Plant Cultivation, Puławy, Poland. Soysaponin 1 (3-Rha-Gal-GlcA-soyasapogenol B) was purified from soybeans as previously described (*28*). The 3-Glc-medicagenic acid was isolated and used as a purified standard (i.e., >98% as determined by HPLC/MS); however, the soyasaponin 1 standard was isolated as a major component (55.7%) in a soyasapogenol B mixture. The purified 3-Glc-medicagenic acid and soyasaponin 1 mixture were used to calculate instrument response curves using negative-ion ESI/HPLC/MS and were highly linear (r^2 > 0.99; **Table 1**). The standard response curves were then used to calculate the absolute concentrations for these two specific compounds and the relative concentrations for 29 other saponins present in *M. truncatula*. Zanhic acid conjugates were identified on the basis of coelution and mass spectrometric properties relative to an authentic standard, 3-Glc-Glc-Glc-23-Ara-28-Ara-Rha-Xyl-Api-zhanic acid, that was purified from alfalfa (*5*, *24*, *25*, *30*, *31*) and kindly provided by W. Oleszek, Institute of Soil Science and Plant Cultivation, Puławy, Poland.

Quantitative analyses were based on the peak area calculated from selected ion chromatograms of the corresponding $[M - H]^-$ ion (**Figure 2**) for standards and quantified saponins with the exception of zhanic acid conjugates, which used the more abundant, doubly charged ion, $[M - 2H]^{2-}$. Absolute and relative quantifications were performed for 31 saponins in total. Relative quantifications for hederagenin, zanhic

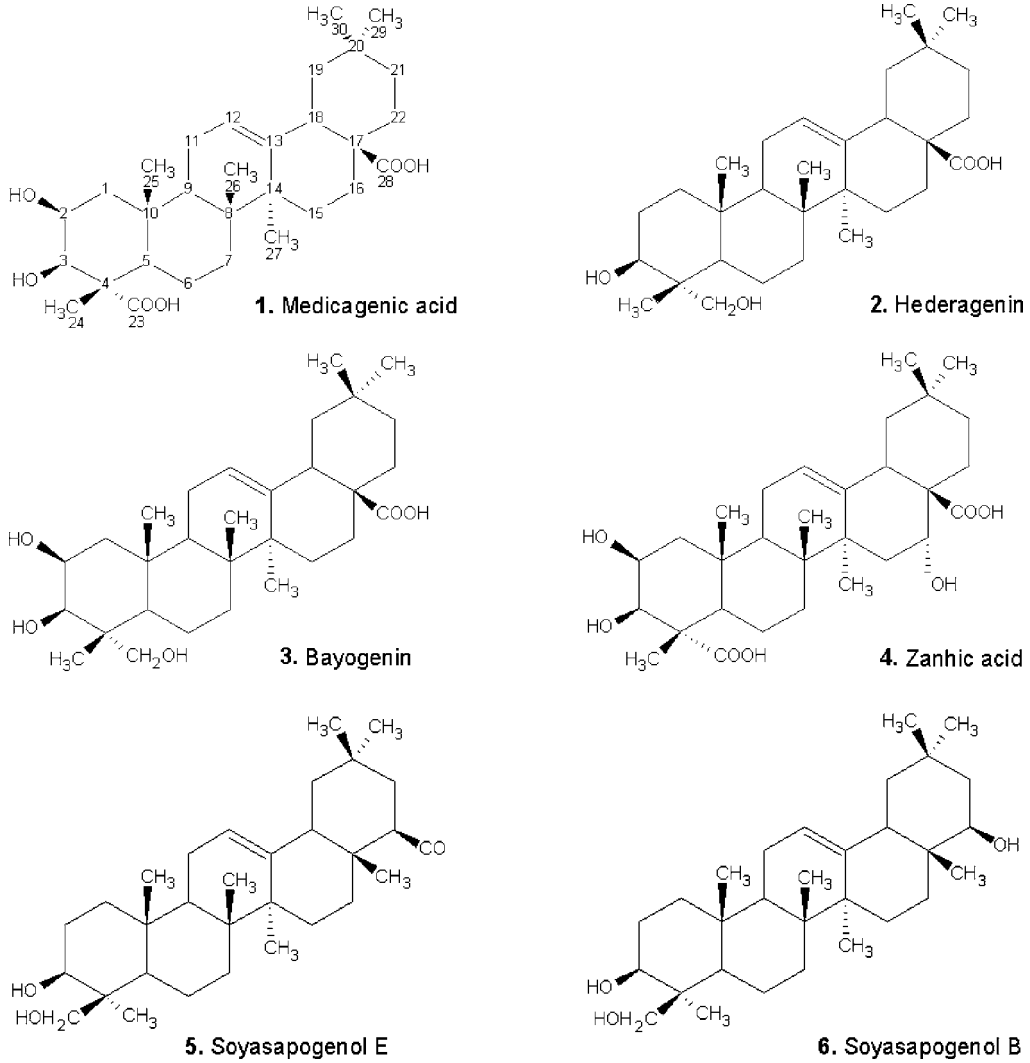

Figure 1. Structures and numbering of the saponin glycosides identified and quantified in *M. truncatula* organs.

acid, bayogenin, and medicagenic acid aglycons and conjugates were calculated on the basis of the linear regression response curve of the 3-Glc-medicagenic acid standard. All relative soyasapogenol aglycon and conjugate quantifications were calculated using the linear regression response curve for the soyasaponin 1 standard. The standard concentration ranges used for quantification of the aerial organs (leaf, stem, seedpod, and seed) were 0.5, 4, and 40 ng/injection of 3-Glc-medicagenic acid and 4, 30, and 200 ng/injection of soyasaponin 1. To accommodate the higher level of saponins in roots, the standard concentration ranges used for roots were 4, 40, and 100 ng/injection of 3-Glc-medicagenic acid and 30, 200, and 400 ng/injection for soyasaponin 1. Standards were prepared in methanol prior to analysis. The saponins quantified consisted of those saponins previously reported (*16*) in addition to zhanic acid conjugates identified through chromatographic and spectrometric comparisons with authentic standards.

An average of eight biological samples, that is, eight individual plants, were sampled and analyzed in duplicate for quantitative calculations with the exception of seeds, in which seeds from five different plants were pooled and sampled due to the limited amount tissue. Duplicate instrumental analyses were performed for both standards and samples with one pass made through the sample set before the duplicate analysis was performed. This was done to limit instrument variation between the first standard and the last sample in the set over a period of 31 h.

Data Analysis. The instrumental response for each of 31 saponins was calculated using Bruker Esquire-LC NT software, version 3.1, to extract peak areas from selected ion chromatograms generated for each molecular ion using an *m*/*z* window of 1 and a retention time window of ±0.75 min. This approach is highly selective and accommodates small drifts in mass and retention times during the experimental time frame. The window criteria were set to be inclusive of actual measured experimental deviation.

RESULTS AND DISCUSSION

An improved method for the separation, quantification, and identification of saponins using HPLC/MS was developed and is a modification of one previously reported (*16*). This method reduced the extraction time from overnight to 2 h while maintaining >95% extraction efficiency as determined by comparison of saponin peak areas of 2 and 24 h extracted material. Furthermore, SPE was eliminated to increase sample throughput by minimizing sample processing time and to lower experimental variance. The method described also utilizes an extended HPLC gradient separation relative to that reported previously (*16*). The extended gradient allows for the combined profiling and identification of other compounds such as isoflavones (*26*, *32*), which normally elute before saponins and are lost during the previously reported SPE method (*16*). Details of the method are provided under Materials and Methods, and the method was used to quantify saponins in various tissues/organs of *M. truncatula*. Representative aglycon structures and a general numbering scheme for the various saponins quantified in this study are provided in **Figure 1**. Representative maximum ion and selected ion chromatograms (SIC) are provided in **Figure 2** and illustrate the diversity of saponins in *M. truncatula*.

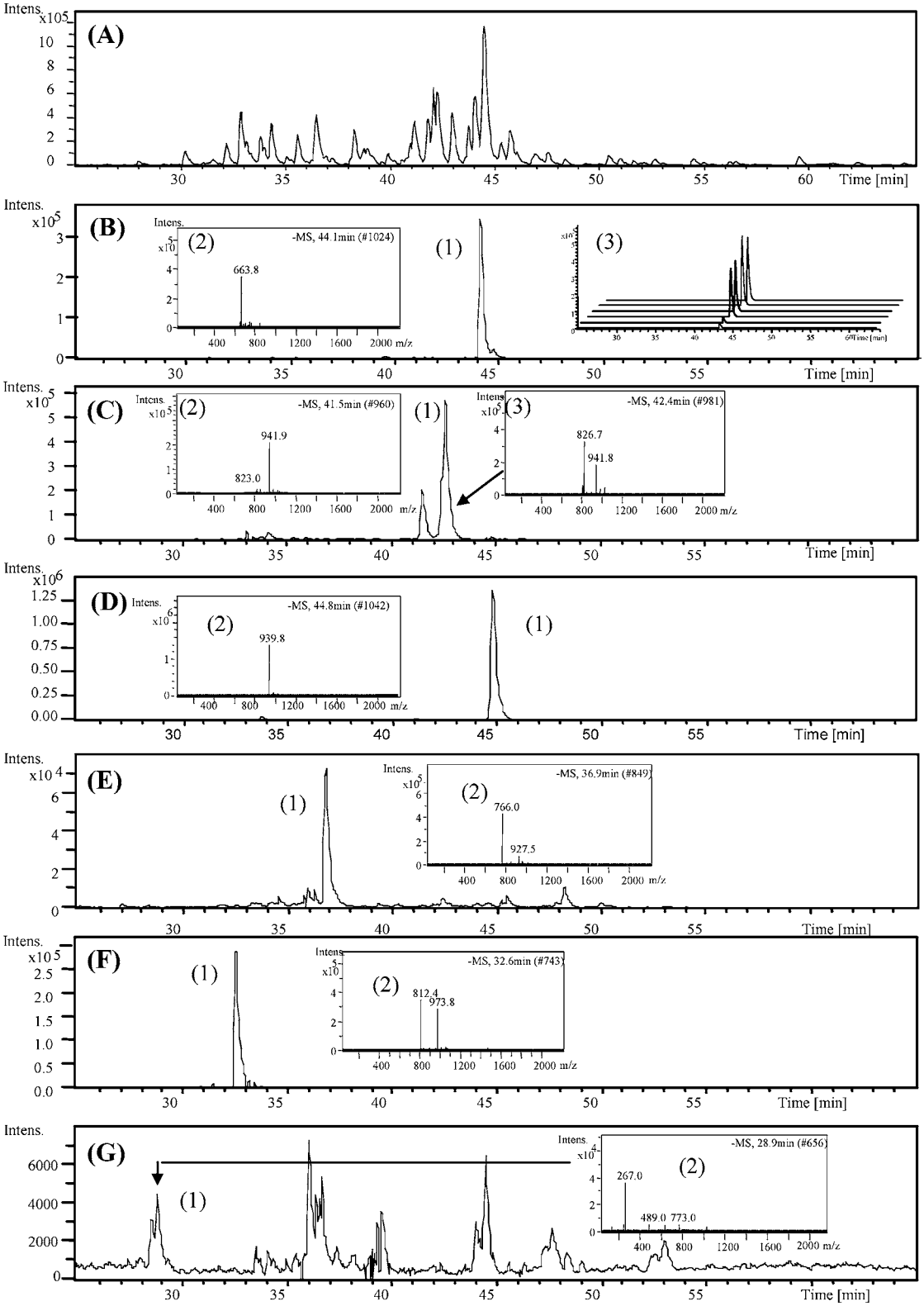

Figure 2. Maximum and selected ion chromatograms for *M. truncatula*, cultivar Jemalong A17, root extracts illustrating the quantitative and qualitative profiling of representative saponins: (**A**) maximum ion chromatogram obtained by negative-ion ESI/HPLC/MS; (**B1**) representative root selected ion chromatogram (*m*/*z* 663, $[M - H]^-$); (**B2**) mass spectrum of 3-Glc-medicagenic acid from root extracts; (**B3**) selected ion chromatograms of replicate standard 3-Glc-medicagenic acid injections from the standard concentration series illustrating the reproducibility and linearity ($r^2 > 0.99$) of the method; (**C1**) selected ion chromatogram (*m*/*z* 941, $[M - H]^-$), mass spectra of (**C2**) Rha-Hex-Hex-hederagenin 1 and (**C3**) soyasaponin 1; (**D1**) selected ion chromatogram (*m*/*z* 939, $[M - H]^-$) and (**D2**) mass spectrum of Rha-Gal-GlcA-soyasapogenol E; (**E1**) selected ion chromatogram (*m*/*z* 927, $[M - H]^-$) and (**E2**) mass spectrum of 3-Glc-Ara-28-Glc-hederagenin; (**F1**) selected ion chromatogram (*m*/*z* 973, $[M - H]^-$) and (**F2**) mass spectrum of Hex-Hex-bayogenin; (**G1**) selected ion chromatogram (*m*/*z* 773 $[M - 2H]^{2-}$) and (**G2**) mass spectrum of 3-Glc-Glc-Glc-23-Ara-28-Ara-Rha-Xyl-zanhic acid observed in *M. truncatula* roots. Peak areas from the SICs were used to calculate relative or absolute concentrations based on the standard response curves of 3-Glc-medicagenic acid or soyasaponin 1.

Table 2. Relative Saponin Concentrations (ng/mg/dw) in Various Tissues of *M. truncatula*[a]

		retention time (t_R)	*m/z* used for quantification $[M - H]^-$	root		stem		leaf		seedpod		seed	
				concn	RSD (%)	concn	RSD (%)	concn	RSD (%)	concn	RSD (%)	concn	RSD (%)
1	3-Glc-Glc-Glc-23-Ara-28-Ara-Rha-Xyl-Api-zanhic acid	29.17	839[b]	0.272	68.1	0.189	47.0	ND	ND	ND	ND	ND	ND
2	3-Glc-Glc-Glc-23-Ara-28-Ara-Rha-Xyl-zanhic acid	29.54	773[b]	3.76	30.5	0.133	40.8	6.45	34.2	0.417	91.3	ND	ND
3	Hex-Hex-Hex-bayogenin	32.23	973	181	39.2	0.972	32.1	0.752	40.9	2.20	124	0.462	58.8
4	3-GlcA-28-AraRhaXyl-medicagenic acid	32.83	1087	5.66	15.2	82.6	20.1	759	14.2	11.2	93.9	869	56.2
5	Rha-Hex-Hex-Hex-hederagenin	33.77	1103	59.7	9.44	1.02	60.7	2.05	52.8	0.316	72.5	0.389	88.0
6	Hex-bayogenin	33.85	649	8.27	24.2	ND	ND	ND	ND	0.258	126	ND	ND
7	3-Glc-28-medicagenic acid	33.98	825	174	41.1	0.621	37.4	ND	ND	ND	ND	3.56	112
8	3-Glc-28-Glc-malonyl-medicagenic acid	34.45	911	219	51.4	0.0762	50.2	0.204	35.6	1.86	100	0.136	50.63
9	3-Glc-28-AraRhaXyl-medicagenic acid	34.53	1073	18.8	13.3	10.7	16.8	1.87	25.1	ND	ND	2.57	91.9
10	Hex-(unknown aglycon)	35.13	647	4.10	54.9	ND	ND	ND	ND	ND	ND	ND	ND
11	Rha-Hex-Hex-Hex-soyasapogenol E	35.62	1087	336	14.5	0.195	83.4	0.401	30.0	5.29	47.7	ND	ND
12	3-Glc-Ara-28-Glc hederagenin	36.53	927	112	18.1	1.85	33.5	15.7	14.3	ND	ND	1.48	82.3
13	Hex-Hex-Rha-bayogenin	40.43	957	3.06	66.2	ND	ND	ND	ND	1.80	93.4	ND	ND
14	Hex-Pen-soyasapogenol E	40.51	749	53.3	34.7	ND	ND	ND	ND	ND	ND	ND	ND
15	Rha-Hex-Hex-hederagenin	41.16	941	283	29.9	0.141	42.9	0.332	74.6	0.133	44.9	0.237	63.8
16	Hex-Hex-bayogenin	42.07	811	291	43.6	ND	ND	2.51	22.7	0.840	60.6	0.118	39.2
17	3-Glc-Glc-medicagenic acid	42.07	825	225	75.3	ND	ND	ND	ND	4.26	119	0.857	149
18	soyasaponin 1[c]	42.26	941	692	37.8	288	9.57	237	33.0	0.628	37.1	60.8	45.7
19	Hex-Hex-(unknown aglycon)	42.98	809	362	67.2	ND	ND	ND	ND	ND	ND	ND	ND
20	Hex-bayogenin (2)	43.28	649	0.333	91.2	ND	ND	ND	ND	ND	ND	ND	ND
21	3-Glc-medicagenic acid[c]	43.75	663	257	18.6	0.141	36.3	ND	ND	0.939	105	2.11	81.8
22	3-Glc-malonyl-medicagenic acid	44.07	749	191	54.0	ND	ND	ND	ND	ND	ND	0.215	62.0
23	Rha-Gal-GlcA-soyasapogenolE	44.46	939	1921	10.6	30.4	13.4	31.7	22.3	0.505	67.3	37.8	47.0
24	Rha-Hex-Hex-soyasapogenol E	44.97	925	84.6	49.4	ND	ND	1.55	41.0	ND	ND	ND	ND
25	Ara-Hex-hederagenin	45.70	765	294	16.5	ND	ND	ND	ND	ND	ND	ND	ND
26	Hex-hederagenin	49.02	633	2.97	38.0	ND	ND	ND	ND	ND	ND	0.200	44.5
27	GlcA-hederagenin	49.28	647	20.5	48.1	ND	ND	ND	ND	ND	ND	ND	ND
28	Hex-Hex-soyasapogenol E	49.71	779	6.48	59.2	ND	ND	4.17	30.6	0.197	17.27	ND	ND
29	Pen-hederagenin	50.93	603	61.9	20.1	ND	ND	ND	ND	ND	ND	ND	ND
30	Hex-hederagenin (2)	51.88	633	24.1	43.8	ND	ND	ND	ND	ND	ND	7.32	32.2
31	Hex-soyasapogenol E	55.36	617	29.5	51.2	ND	ND	ND	ND	ND	ND	ND	ND
	total saponin and av RSD per organ			**5924**	*39.9*	**417**	*37.4*	**1064**	*33.6*	**30.8**	*80.0*	**991**	*69.1*

[a] All relative concentrations were calculated based on peak areas of the $[M - H]^-$ selected ion chromatorgrams relative to the standard soyasaponin 1 (soyasaponins B and E conjugates) or 3-Glc-medicagenic acid (bayogenin, hederagenin, medicagenic acid conjugates). Abbreviations: Api, apiofuranose; Ara, arabinose; Gal, galactose; Glc, glucose; GlcA, galacturonic acid; Hex, hexose; Pen, pentose; Rha, rhamnose; Xyl, xylose; concn, concentration; ND, not detected or below level of detection; ng/mg of dw, nanograms per milligram of dry weight; RSD, relative standard deviation of saponin concentrations measured for eight independent plants. [b] $[M - 2H]^{2-}$ ion used for quantification of zanhic acid conjugates. [c] Absolute concentrations reported for soyasaponin 1 and 3-Glc-medicagenic acid (i.e., same as **Table 1**).

The reported method was used to quantitatively profile 31 saponins in *M. truncatula* and is more comprehensive than that previously reported (*16*). Additional saponins profiled using this method included zanhic acids, which to the best of the authors' knowledge have not been reported in the roots of *M. truncatula* previously.

Absolute and relative quantifications of saponins were performed using the standards 3-Glc-medicagenic acid purified from alfalfa (*24*, *30*) and soyasaponin 1 (3-Rha-Gal-GlcA-soyasapogenol B), which was purified from soybeans (*28*). Response curves were generated for the standard saponins and used to calculate the absolute concentrations for these two specific compounds present in *M. truncatula* (**Table 1**). Absolute quantification of all saponins in *M. truncatula* was not possible due to the limited number of available saponin standards; therefore, relative quantification was performed for 29 other saponins in *M. truncatula* (**Table 2**). All relative quantifications were performed on the basis of the similarity of the aglycon structures seen in **Figure 1** to those of the representative standards. An average of eight biological samples, that is, individual plants, were sampled, and duplicate instrumental analyses were performed on each.

To allow the statistical assessment of the quantitative results, relative standard deviations (RSD) for both instrumental and biological (plant-to-plant) variability were investigated. An average instrumental/analytical coefficient of variation (relative standard deviation) was calculated for all of the components represented in seven replicated injections of an alfalfa (cv. Radius 4) saponin extract analyzed by negative-ion ESI/HPLC/MS and determined to be 12.4% on the basis of variations in peak areas using software integration and 9.8% by measuring relative intensities (*33*). This value is similar to other reported LC/MS methods for the quantification of saponins in soybean (*26*). In addition, biological variability was also quantified. Biological variability is defined as the quantitative physiological difference observed in different individual plants (i.e., plant-to-plant) generally attributed to small differences in their microenvironments or developmental stage (*34*). Although the analytical or instrumental variation of the described LC/MS method was relatively low (RSD = 12%), the variability of the biological system was significantly higher. The average biological coefficient of variation associated with saponin accumulation in various tissues of *M. truncatula* was (A) roots, 40%; (B) stems, 37%; (C) leaves, 34%; (D) seedpods, 80%; and (E) seeds, 69%. The coefficients of variation of roots, stem, and leaves are similar to the 50% previously reported for *M. truncatula* and other plants (*32*, *33*, *35*). The higher biological coefficients of variation reported for seed and seedpod are attributed to the fact that the sampled seed and seedpods were of different developmental stages.

Significant quantitative and qualitative differences in saponin profiles were observed in the different tissues of *M. truncatula* (**Table 2**). Roots had the highest relative total saponin content measured at 5.92 μg/mg of dw. This amount is approximately 5 times that in leaves and seed, 10 times that in stems, and approximately 200 times that in seed pods. Roots also contained the greatest diversity of saponins with 31 different saponin conjugates quantified. Leaves contained the next highest number of different saponins, numbering 17; however, several saponins such as 3-Glc-28-Glc-malonyl medicagenic acid (0.203 ng/mg of dw) were observed in only trace amounts. The relative total saponin contents of *M. truncatula* were as follows: leaves, 1.06 μg/mg of dw; seed, 0.991 μg/mg of dw; stem, 0.417 μg/mg of dw; and seedpod, 0.031 μg/mg of dw. The relative total saponin content measured for *M. truncatula* roots is similar to those reported for the alfalfa (*Medicago sativa*) cultivars Radius at 5 μg/mg of dw (*30*) and Kleszczewska at 9.33 μg/mg ofdw (*36*). Although *M. truncatula* roots had a relative total saponin content similar to that of alfalfa roots, the relative total saponin content in *M. truncatula* leaves was lower than those reported for the leaves of the alfalfa cultivars Resis (4 μg/mg of dw) and Lahontan (2 μg/mg of dw) (*37*) and significantly lower than those in the leaves and stem of the alfalfa variety Kleszczewska (30 μg/mg of dw) (*38*).

The quantitative data reveal organ specific and differential accumulation of triterpene saponins. In the roots of *M. truncatula*, the major saponins were soyasapogenol conjugates, with >32% of the relative total saponin content being present as Rha-Gal-GlcA-soyasapogenol E. Conversely in leaf, seed, and seedpod, the major constituents were medicagenic acid conjugates. More specifically, the major component of leaf was 71% 3-GlcA-28-Ara-Rha-Xyl-medicagenic acid. The differential accumulation of medicagenic acid conjugates in *M. truncatula* leaf is consistent with other reports that cite medicagenic acid as the dominant aglycon in the aerial parts of *Medicago* species (*19*) and with more recent molecular genetic expression patterns of saponin glucosyltransferases in *M. truncatula* (*39*).

The differential accumulation of medicagenic acid conjugates in leaf is most likely the result of spatially resolved biosynthesis that is targeted toward specific plant defense mechanisms such as antiherbivory. This is consistent with reports that associate medicagenic acids with repelling insects, bloating in ruminants, growth retardation of nonruminants, and hemolytic activities (*10*, *11*, *40*). This hypothesis is further supported by reports that the level of 3-GlcA-28-Ara-Rha-Xyl-medicagenic acid doubled in insect-damaged foliage of alfalfa (*40*) and that 3-GlcA-28-Ara-Rha-Xyl-medicagenic acid is the predominant biologically active saponin in aerial parts of alfalfa based upon the *T. viride* test (*9*). Specific conjugation patterns may also be indicative of function as the level of biological activity of saponins is often associated with specific sugar composition, and in cereals, the removal of a single sugar unit can result in reduction of this activity (*2*). Similarly, the differential accumulation of soyasapogenols in roots may be indicative of specific functions such as plant−microbe signaling (*41*, *42*) or alleopathic interactions (*11*, *43*, *44*).

ABBREVIATIONS USED

Api, apiofuranose; Ara, arabinose; av, average; Gal, galactose; Glc, glucose; GlcA, galacturonic acid; Hex, hexose; ng/mg of dw, nanograms per milligram of dry weight; Pen, pentose; PDA, photodiode array; Rha, rhamnose; SPE, solid-phase extraction; Xyl, xylose.

ACKNOWLEDGMENT

We thank Wieslaw Oleszek, Institute of Soil Science and Plant Cultivation, Puławy, Poland, for providing authentic standards cited in a previous report (*16*) and for the 3-Glc medicagenic acid and 3-Glc-Glc-Glc-23-Ara-28-Ara-Rha-Xyl-Api-zanhic acid reported in this study. We also thank Dr. Aldo Tava, Instituto Sperimentale per le Colture Foraggere, Italy, for providing additional authentic saponins.

LITERATURE CITED

(1) Hostettmann, K.; Marston, A. *Saponins*; Cambridge University Press: New York, 1995; p 548.
(2) Osbourn, A. E. Saponins in Cereals. *Phytochemistry* **2003**, *62*, 1−4.
(3) Xu, R.; Fazio, G. C.; Matsuda, S. P. T. On the origins of triterpenoid skeletal diversity. *Phytochemistry* **2004**, *65*, 261−291.
(4) Osbourn, A. E. Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell* **1996**, *8*, 1821−1831.
(5) Tava, A.; Odoardi, M. Saponins from *Medicago* spp.: Chemical characterization and biological activity against insects. In *Saponins Used in Food and Agriculture*; Waller, G., Yamasaki, K., Eds.; Plenum Press: New York, 1996; pp 97−109.
(6) Oleszek, W. Structural specificity of alfalfa (*Medicago sativa*) saponin haemolysis and its impact on two haemolysis-based quantification methods. *J. Sci. Food Agric.* **1990**, *53*, 477−485.
(7) Nagata, T.; Tsushida, T.; Hamaya, E.; Enoki, N.; Manabe, S.; Nishino, C. Camellidins, antifungal saponins isolated from *Camellia japonica*. *Agric. Biol. Chem.* **1985**, *49*, 1181−1186.
(8) Oleszek, W. Alfalfa saponins: Structure, biological activity, and chemotaxonomy. In *Saponins Used in Food and Agriculture*, *Advances in Experimental Medicine and Biology*; Waller, G., Yamasaki, K., Eds.; Plenum Press: New York, 1996; pp 155−170.
(9) Sen, S.; Makkar, H. P. S.; Becker, K. Alfalfa saponins and their implication in animal nutrition. *J. Agric. Food Chem.* **1998**, *46*, 131−140.
(10) *Saponins Used in Traditional and Modern Medicine*; Waller, G. R., Yamasaki, K., Eds.; Plenum Press: New York, 1996; p 606.
(11) *Saponins Used in Food and Agriculture*; Waller, G. R., Yamasaki, K., Eds.; Plenum Press: New York, 1996; p 441.
(12) Haridas, V.; Higuchi, M.; Jayatilake, G. S.; Bailey, D.; Mujoo, K.; Blake, M. E.; Arntzen, C. J.; Gutterman, J. U. Avicins: Triterpenoid saponins from *Acacia victoriae* (Bentham) induce apoptosis by mitochondrial perturbation. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 5821−5826.
(13) Wu, C. A.; Yang, Y. W. Induction of cell death by saponin and antigen delivery. *Pharm. Res.* **2004**, *21*, 271−277.
(14) Sun, H. X.; Pan, H. J.; Pan, Y. J. Haemolytic activities and immunologic adjuvant effect of *Panax notoginseng* saponins. *Acta Pharmacol. Sin.* **2003**, *24*, 1150−1154.
(15) Kirk, D. D.; Rempel, R.; Pinkhasov, J.; Walmsley, A. M. Application of *Quillaja saponaria* extracts as oral adjuvants for plant-made vaccines. *Expert Opin. Biol. Ther.* **2004**, *4*, 947−958.
(16) Huhman, D.; Sumner, L. Metabolic profiling of saponins in *Medicago sativa* and *Medicago truncatula* using HPLC coupled to an electrospray ion-trap mass spectrometer. *Phytochemistry* **2002**, *59*, 347−360.
(17) Suzuki, H.; Achnine, L.; Xu, Y.; Matsuda, S. P.; Dixon, R. A. A genomics approach to the early stages of triterpene saponin biosynthesis in *Medicago truncatula*. *Plant J.* **2002**, *32*, 1033−1048.
(18) Pérez, N.; Pena, S.; Vega, S.; Noa, M.; Enríquez, R. Medicagenic acid content in foliage of ten varieties of alfalfa (*Medicago sativa* L.) cultivated in Mexico. *J. Sci. Food Agric.* **1997**, *75*, 401−404.

(19) Jurzysta, M.; Waller, G. R. Antifungal and hemolytic activity of aerial parts of alfalfa (*Medicago*) species in relation to saponin composition. In *Saponins Used in Traditional and Modern Medicine, Advances in Experimental Medicine and Biology*; Waller, G. R., Yamasaki, K., Eds.; Plenum Press: New York, 1996; pp 565−574.

(20) Oleszek, W. Chromatographic determination of plant saponins. *J. Chromatogr. A* **2002**, *967*, 147−162.

(21) Domon, B.; Dorsaz, A.-C.; Hostettmann, K. High-performance liquid chromatography of oleanane saponins. *J. Chromatogr.* **1984**, *315*, 441−446.

(22) Burnouf-Radosevich, M.; Delfel, N. E. High-performance liquid chromatography of triterpene saponins. *J. Chromatogr.* **1986**, *368*, 433−438.

(23) Hu, J.; Lee, S.-O.; Hendrich, S.; Murphy, P. A. Quantification of the group B soyasaponins by high-performance liquid chromatography. *J. Agric. Food Chem* **2002**, *50*, 2587−2594.

(24) Oleszek, W.; Price, K. R.; Colquhoun, I. J.; Jurzysta, M.; Ploszynski, M.; Fenwick, G. R. Isolation and identification of alfalfa (*Medicago sativa* L.) root saponins: Their activity in relation to a fungal bioassay. *J. Agric. Food Chem.* **1990**, *38*, 1810−1817.

(25) Nowacka, J.; Oleszek, W. High performance liquid chromatography of zanhic acid glycoside in alfalfa. *Phytochem. Anal.* **1992**, *3*, 227−230.

(26) Dalluge, J.; Eliason, E.; Frazer, S. Simultaneous identification of soyasaponins and isoflavones and quantification of soyasaponin Bb in soy products, using liquid chromatography/electrospray ionization-mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 3520−3524.

(27) Gu, L.; Tao, G.; Gu, W.; Prior, R. Determination of soyasaponins in soy with LC-MS following structural unification by partial alkaline degradation. *J. Agric. Food Chem.* **2002**, *50*, 6951−6959.

(28) Berhow, M. A.; Cantrell, C. L.; Duval, S. M.; Dobbins, T. A.; Maynes, J.; Vaughn, S. F. Analysis and quantitative determination of group B saponins in processed soybean products. *Phytochem. Anal.* **2002**, *13*, 343−348.

(29) Oleszek, W. Solid-phase extraction-fractionation of alfalfa saponins. *J. Sci. Food Agric.* **1988**, *44*, 43−49.

(30) Bialy, Z.; Jurzysta, M.; Oleszek, W.; Piacente, S.; Pizza, C. Saponins in alfalfa (*Medicago sativa* L.) root and their structural elucidation. *J. Agric. Food Chem.* **1999**, *47*, 3185−3192.

(31) Nowacka, J.; Oleszek, W. Determination of alfalfa (*Medicago sativa*) saponins by high-performance liquid chromatography. *J. Agric. Food Chem.* **1994**, *42*, 727−730.

(32) Broeckling, C. D.; Huhman, D. V.; Farag, M. A.; Smith, J. T.; May, G. D.; Mendes, P.; Dixon, R. A.; Sumner, L. W. Metabolic profiling of *Medicago truncatula* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. *J. Exp. Bot.* **2005**, *56*, 323−336.

(33) Duran, A. L.; Yang, J.; Wang, L.; Sumner, L. W. Metabolomics spectral formatting, alignment and conversion tools (MSFACTs). *Bioinformatics* **2003**, *19*, 2283−2293.

(34) Asirvatham, V.; Watson, B.; Sumner, L. Analytical and biological variances associated with proteomic studies of *Medicago truncatula* by two-dimensional polyacrylamide gel electrophoresis. *Proteomics* **2002**, *2*, 960−968.

(35) Fiehn, O.; Kopka, J.; Dörmann, P.; Altmann, T.; Trethewey, R. N.; Willmitzer, L. Metabolic profiling for plant functional genomics. *Nat. Biotechnol.* **2000**, *18*, 1157−1161.

(36) Oleszek, W.; Jurzysta, M.; Price, K. R.; Fenwick, G. R. High-performance liquid chromatography of alfalfa root saponins. *J. Chromatogr.* **1990**, *519*, 109−116.

(37) Massiot, G.; Lavaud, C.; Besson, V.; Le Men-Olivier, L.; Van Binst, G. Saponins from aerial parts of alfalfa (*Medicago sativa* L.). *J. Agric. Food Chem.* **1991**, *39*, 78−82.

(38) Oleszek, W.; Jurzysta, M.; Ploszynski, M.; Colquhoun, I. J.; Price, K. R.; Fenwick, G. R. Zanhic acid tridesmoside and other dominant saponins from alfalfa (*Medicago sativa* L.) aerial parts. *J. Agric. Food Chem.* **1992**, *40*, 191−196.

(39) Achnine, L.; Huhman, D.; Farag, M.; Sumner, L. W.; Blount, J. W.; Dixon, R. A. Genomics-based identification of triterpene glycosyltransferases from the model legume *Medicago truncatula*. *Plant J.* **2005**, in press (doi 10.1111/j.1365-313X.2005.02344.x).

(40) Agrell, A.; Oleszek, W.; Stochmal, A.; Olsen, M.; Anderson, P. Herbivore-induced response in alfalfa (*Medicago sativa*). *J. Chem. Ecol.* **2003**, *29*, 303−320.

(41) Oleszek, W.; Hoagland, R. E.; Zablotowicz, R. M. Ecological significance of plant saponins. In *Principles and Practices of Plant Ecology. Alleochemical Interactions*; Inderjit Dakshini, K. M. M., Foy, C. L., Eds.; CRC Press: Boca Raton, FL, 1999; pp 451−465.

(42) Oleszek, W. A.; Stochmal, A. Triterpene saponins and flavonoids in the seeds of *Trifolium* species. *Phytochemistry* **2002**, *61*, 165−170.

(43) Lynn, D. G. The involvement of allelochemicals in the host selection of parasitic angiosperms. In *The Chemistry of Allelopathy, Biochemical Interactions Among Plants*; Thompson, A. C., Ed.; American Chemical Society: Washington, DC, 1985; pp 55−81.

(44) Waller, G. R.; Jurzysta, M.; Thorne, R. L. Z. Allelopathic activity of root saponins from alfalfa (*Medicago sativa* L.) on weeds and wheat. *Bot. Bull. Acad. Sin.* **1993**, 1−11.

Received for review October 19, 2004. Revised manuscript received January 12, 2005. Accepted January 12, 2005. This work was financially supported by The Samuel Roberts Noble Foundation.

JF0482663