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Metabolic profiling of saponins in *Medicago sativa* and *Medicago truncatula* using HPLC coupled to an electrospray ion-trap mass spectrometer

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Abstract

Triterpene saponins isolated from *Medicago sativa* (alfalfa) and *Medicago truncatula* roots were separated, profiled and identified using an optimized, reversed-phase HPLC with on-line photodiode array detection and electrospray ionization mass spectrometry method (HPLC/PDA/ESI/MS). ESI source polarity and solvent conditions were compared. The effects of these parameters on mass spectral attributes were determined. Ion structures were confirmed using tandem mass spectrometry (MS/MS). Fifteen saponins were identified in alfalfa (cultivars Apollo, Radius, and Kleszczewska) based upon negative-ion HPLC/PDA/ESI/MS, HPLC/PDA/ESI/MS/MS and literature data. In addition, the identification of two new malonated saponins in alfalfa are proposed. Negative-ion HPLC/PDA/ESI/MS and HPLC/PDA/ESI/MS/MS spectra were utilized along with HPLC retention times to profile and identify 27 saponins in *M. truncatula* (cultivar Jemalong, A17). *M. truncatula* yielded a much more complex mixture of saponins than observed for alfalfa. The authors are not aware of any previous reports identifying saponin glycosides in *M. truncatula*. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Medicago sativa; Alfalfa; *Medicago truncatula*; Fabaceae; Legumes; Metabolite profiling; Saponins; Malonyl saponins; High performance liquid chromatography (HPLC); Electrospray ionization (ESI); Mass spectrometry (MS)

1. Introduction

Legumes are important agricultural and commercial crops characterized by root nodules formed as a result of the symbiotic relationship with nitrogen-fixing rhizobia. The Leguminosae taxonomic class includes plants such as soybean (*Glycine max*), which provides a major source of protein and oil for both humans and animals, and alfalfa (*Medicago sativa*) that serves as an important forage and soil conditioning crop (Cook, 1999). *Medicago truncatula*, a close relative of alfalfa, has been chosen as a model legume because of its prolific nature, its small diploid genome ($\sim 5\times10^8$ bp), self-fertilization, ease of genetic transformation, and rapid generation time (Cook, 1999; Trieu et al., 2000). We have targeted *M. truncatula* in a functional genomics project that incorporates profiling of gene expression (transcriptome), protein expression (proteome), and metabolite expression (metabolome) to better understand the biological processes associated with legumes and their interaction with their environment (Bell et al., 2000). Profiling and identification of a large variety of natural products from *M. truncatula*, including saponins, is crucial to metabolomics and functional genomics (Trethewey et al., 1999; Fiehn et al, 2000). Due to the chemically complexity of the metabolome there does not exist any singular technique for profiling all of the metabolites simultaneously. Our approach to metabolomics involves segregation of the metabolome into several subclasses followed by parallel analyses utilizing the selectivity of mass spectrometry. This paper reports on the method development and chemical characterization of one of the more challenging subclasses, the saponins, in *M. sativa* and *M. truncatula*.

Abbreviations: ESI, electrospray ionisation; SPE, solid phase extraction; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PDA, photodiode array; Glc, β-D-glucopyranosyl; Gal, β-D-galactopyranosyl; GlcA, β-D-glucuronopyranosyl; Rha, α-L-rhamnopyranosyl; Xyl, β-D-xylopyranosyl; Ara, α-L-arabinopyranosyl

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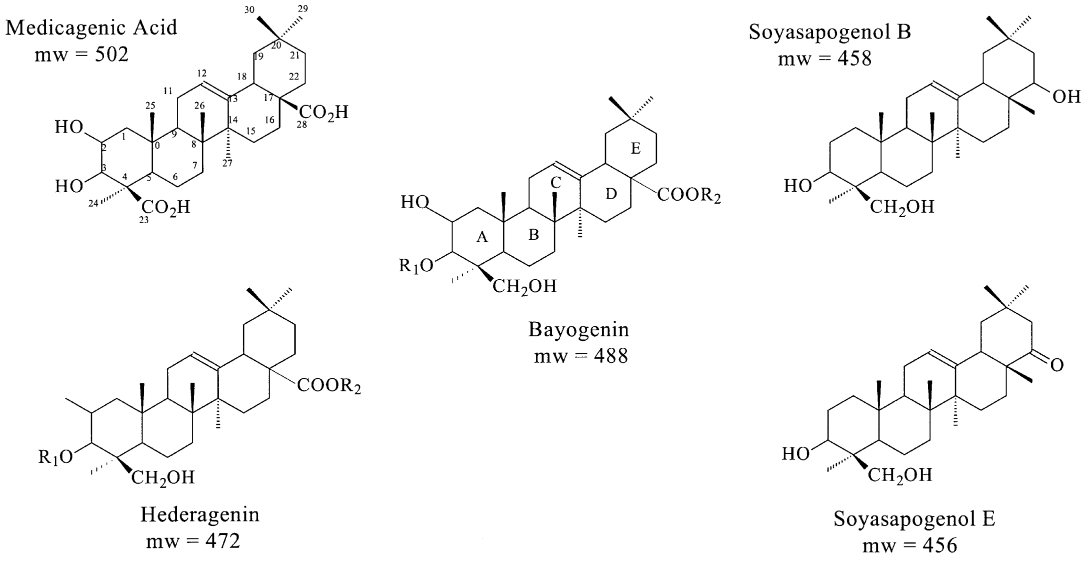
Saponins consist of triterpenoid or steroidal aglycones that are substituted with a varying number of sugar side chains. The unsubstituted, nonpolar aglycones are classified as sapogenins. Structures for common legume sapogenins are provided in Fig. 1. These compounds have been associated with a variety of biological activities including: allelopathy (i.e. competitive chemical interactions between plants such as secretion into the soil resulting in low seed germination rates; Waller et al., 1993), poor digestibility in ruminants (Oleszek, 1996), deterrence to insect foraging (Tava and Odoardi, 1997) and beneficial antifungal properties (Nagata et al., 1985). Further, plant species containing saponins have long been used in folk medicine and have been shown to possess anti-inflammatory, hemolytic, cholesterol lowering, and anticancer properties (Jurzysta and Nowacki, 1979; Malinow et al., 1982; Waller and Yamasaki, 1996a). More recently, saponins isolated from the legume, *Acacia victoriae*, have been reported to trigger apoptosis in cancer cells (Haridas et al., 2001). Based on the above properties, the presence and role of saponins in alfalfa and other plant systems have been the subject of intense study.

Traditionally, saponins have been extracted, separated by thin layer chromatography (TLC) or liquid chromatography and then analyzed by MS in a batch mode. Recently, a cumulative report identifying 24 saponin conjugates in alfalfa using this approach was presented (Bialy et al., 1999). Separation and profiling of saponins using HPLC offers enhanced chromatographic resolution but detection is complicated by the fact that saponins contain poor chromophores. HPLC detection techniques using refractive index (Nagasawa et al., 1980), UV absorbance at 206 nm (Domon et al., 1984; Burnouf-Radosevich and Delfel, 1986) and light scattering (Ireland and Dziedzic, 1986) detection have been reported but suffer from low sensitivity in addition to restrictive solvent and gradient conditions. Improved sensitivity has been achieved through derivatization using 4-bromophenacyl bromide (Slacanin et al., 1988) prior to HPLC analysis and was demonstrated for the analysis of alfalfa root saponins (Oleszek et al., 1990a; Nowacka and Oleszek, 1992). These reports described linear responses within the range of 1–15 μg, but also noted that the derivatized standards decomposed at room temperature after 48 h.

A simplified, sensitive, and selective analysis alternative is HPLC coupled to mass selective detection. A variety of mass spectrometric techniques used for the structural analysis and characterization of saponins have been presented and summarized (Waller and Yamasaki, 1996b). Fang et al. (1998, 1999) illustrated the use of ESI and tandem mass spectrometry in the structural elucidation of saponins in a perennial trailing plant (*Tribulus terrestris*) extract introduced by constant infusion into an ion trap mass spectrometer. Perret et al. (1999) coupled negative-ion ESI to HPLC/MS/MS for the analysis of saponins in the dried berries of an Ethiopian plant, endod (*Phytolacca dodecandra*), and compared many instrumental parameters. They also discussed post column addition of various mobile phase modifiers including ammonium hydroxide for enhanced negative ion generation. Finally, tandem MS has also been utilized in the structural characterization of saponins from *Quillaja saponaria* bark extracts (van Setten et al., 1998, 2000).

We report here a multidimensional HPLC/PDA/ESI/MS/MS method that was developed and optimized for metabolic profiling of saponin conjugates in legumes. This method utilized HPLC with both on-line UV and


Medicagenic Acid
mw = 502

Soyasapogenol B
mw = 458

Bayogenin
mw = 488

Hederagenin
mw = 472

Soyasapogenol E
mw = 456

Fig. 1. Chemical structures and numbering scheme of common saponin aglycones found in legumes.

mass selective detection. Further, automated and data dependent tandem mass spectrometry (MS/MS) was performed to assist in structural elucidation. The method was utilized to differentiate, identify and compare saponins in *M. sativa* and *M. truncatula* root extracts.

2. Results and discussion

2.1. Method optimisation

The goals of this project were to establish and optimize a HPLC/MS method for the metabolic profiling of saponins in *M. truncatula*. We also desired to qualitatively differentiate and identify a significant number of these compounds. These goals were complicated by the absence of commercial standards and literature reports of saponin glycosides in *M. truncatula*. Fortunately, the literature contains detailed structural and identity information on the sapogenin and saponin content in alfalfa, a close relative of *M. truncatula*. Given the quantity of information available for alfalfa and to overcome the absence of standards, we chose to use purified model compounds from alfalfa to optimize our HPLC/MS and HPLC/MS/MS profiling method. Initial optimization experiments were performed using model saponin compounds purified from alfalfa while profiling experiments were performed using fractionated extracts (Section 4). HPLC/MS analyses of alfalfa model compounds and extracts served as positive controls and were performed to record HPLC retention times and characterize the mass spectra of known saponins in alfalfa. The literature is referenced to provide supporting structural conformation. The patterns observed during the HPLC/MS and HPLC/MS/MS analysis of alfalfa saponins were then extrapolated to the profiling and identification of saponins in *M. truncatula*.

The effect of solution pH and instrument polarity on saponin ionization efficiency was evaluated first. This was performed through comparisons of positive and negative-ion ESI-MS spectra of the model compound, 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl (1→2)-β-D-glucuronopyranoside] soyasapogenol B (comomon name, soysaponin I, mw 942) obtained from various solutions. These results are summarized in Fig. 2. Both acidic and basic solutions were investigated based on the ability of these solutions to enhance ion formation, i.e. protonation or deprotonation.

The highest absolute instrument response for soyasaponin I was obtained using negative-ion ESI and a basic NH_4OH solution (Fig. 2c). This is in agreement with a recent report presented by Perret et al. (1999). The ion abundances recorded for the acetic acid solutions in both positive and negative-ion modes were lower by a factor of four and six respectively. Although the negative-ion ESI–MS spectra yielded a $[M–H]^-$ anion at approximately half the absolute abundance of that observed for the $[M+H]^+$ cation in the acetic acid solution, the negative-ion spectra had a significantly higher signal-to-noise ratio (400:1) than that recorded in positive-ion mode (133:1; Fig. 2). The greater signal-to-noise ratio for the negative-ion ESI/HPLC/MS is interpreted as greater sensitivity even though the positive-ion mode yielded higher absolute ion abundances when using acetic acid mobile phases.

The positive-ion ESI mass spectra obtained using a 0.1% acetic acid mobile phase were characterized by ions corresponding to cation attachment to the molecular species, (i.e. $[M+H]^+$, $[M+Na]^+$) and several lower *m/z* fragment ions. These fragment ions correlate to sequential loss of sugar substituents and thus the positive-ion spectra contain significantly more structural information than observed in the negative-ion mode. Negative-ion ESI mass spectra of the saponin glycosides obtained under exact instrumental conditions with the exception of voltage polarity, were relatively simple when compared to their positive-ion ESI mass spectra. The negative-ion mass spectra yielded strong $[M–H]^-$ and $[2M–H]^-$ ions that allow easy confirmation of molecular weight. Negative fragment ions corresponding to the sequential loss of sugars were observed but at dramatically lower abundances than those observed in the positive-ion mode. Increased structural information could be obtained in the negative-ion ESI/MS mode by increasing the capillary-skimmer voltage offset or using tandem MS. Conversely, the amount of in-source fragmentation observed in the positive-ion ESI/MS mode could be reduced by lowering the capillary-skimmer voltage offset, but this often resulted in incomplete solvent removal (Bruins, 1997).

Negative-ion ESI studies also showed that elevated pH values resulted in an increased fragment ion at *m/z* 439 for 3-*O*-β-D-glucopyranosyl-medicagenic acid, an abundant *M. sativa* saponin. This characteristic ion is the result of base catalyzed hydrolysis of the sugar substituent followed by decarboxylation and dehydration of the medicagenic acid aglycone $[M–Glc–CO_2–H_2O–H]^-$. The *m/z* 439 has previously been reported as a fragment ion associated with medicagenic acid conjugates (West et al., 1996). The relative intensity of the decarboxylation/dehydration ion increased as the pH increased (see Fig. 3) thus showing a direct dependence. Fig. 3 further reiterates the effect of solution pH on the increased negative-ion abundance with 3-Glc-medicagenic acid. The negative-ion abundance is observed to increase by a factor of four as the pH increases from pH 3.5 to 8.5, similar to that observed in Fig. 2 and for soyasaponin I. The instrument response was determined to reach a maximum in the pH range of 8.0 to 8.5.

The data presented in Figs. 2 and 3 confirm that negative-ion ESI with NH_4OH yields maximum MS instrumental response for saponins in a flow-injection or batch mode; however an acidic environment is preferred due to enhanced HPLC chromatographic resolution and efficiency for acidic saponins. Acidic or lower pH solutions ensure that the carboxy acid substituents remain protonated to maximize hydrophobic interactions with the stationary phase. The effect of mobile phase modifiers and pH on the HPLC separation efficiency (N) of 3-Glc-medicagenic acid was evaluated using various mobile phase modifiers including formic acid, propanoic acid, acetic acid, and trifluoroacetic acid, ammonium acetate buffered to pH 7.0 with NH_4OH, and MilliQ water. The results are summarized in Table 1 and show an improvement in separation efficiency for 3-Glc-medicagenic acid with the acidic mobile phases. The utility of a trifluoroacetic acid modifier on the HPLC separation on triterpene sapononins has been reported. (Burnouf-Radosevich and Delfel, 1986). Unfortunately, TFA yielded a lower separation efficiency and a lower ESI/MS instrument response for 3-Glc-medicagenic acid due to increased ion pair energetics compared to other organic acids (Wang and Cole, 1996). It was concluded that a mobile phase of 0.1% acetic acid was best based upon its enhanced chromatographic efficiency and ESI/MS instrument response.

Although better chromatographic separation efficiencies are achieved in acidic mobile phases, improved ESI/MS response is achieved using slightly basic solutions.

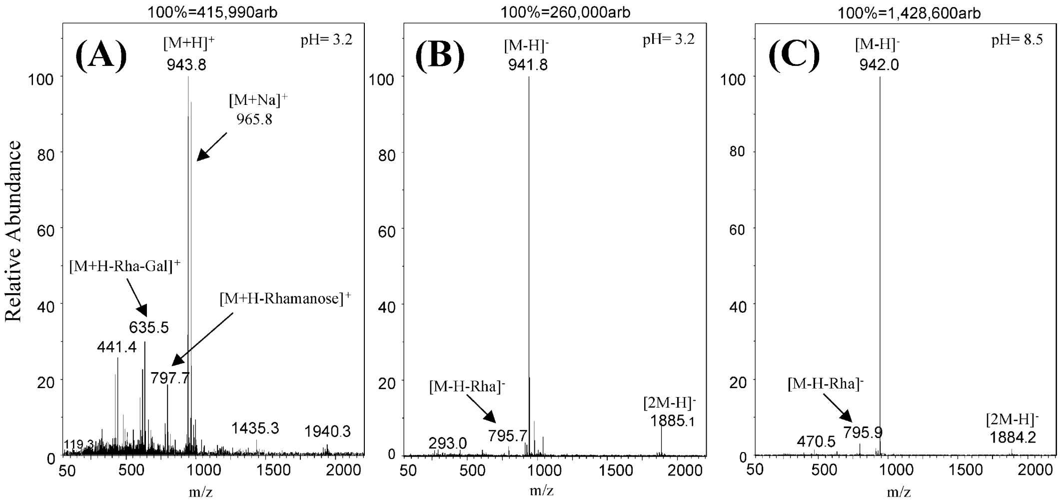


Fig. 2. Comparison of instrument polarity and solution pH on the ion abundance of 200 ng injections of soyasaponin I. Instrumental responses were normalized to 100% but absolute responses in arbitrary units are provided in the heading of each spectra. Soyasaponin I was flow-injected into a mobile phase of 50:50 H_2O:CH_3CN and mass spectra recorded using (A) positive-ion ESI/MS, 0.1% acetic acid, pH = 3.5, S/N of $[M+H]^+ = 133$ (B) negative-ion ESI/MS, 0.1% acetic acid, pH = 3.5, S/N of $[M\text{-}H]^- = 400$ and (C) negative-ion ESI/MS, 125 μM NH_4OH, pH = 8.5, S/N of $[M–H]^- = 3333$.

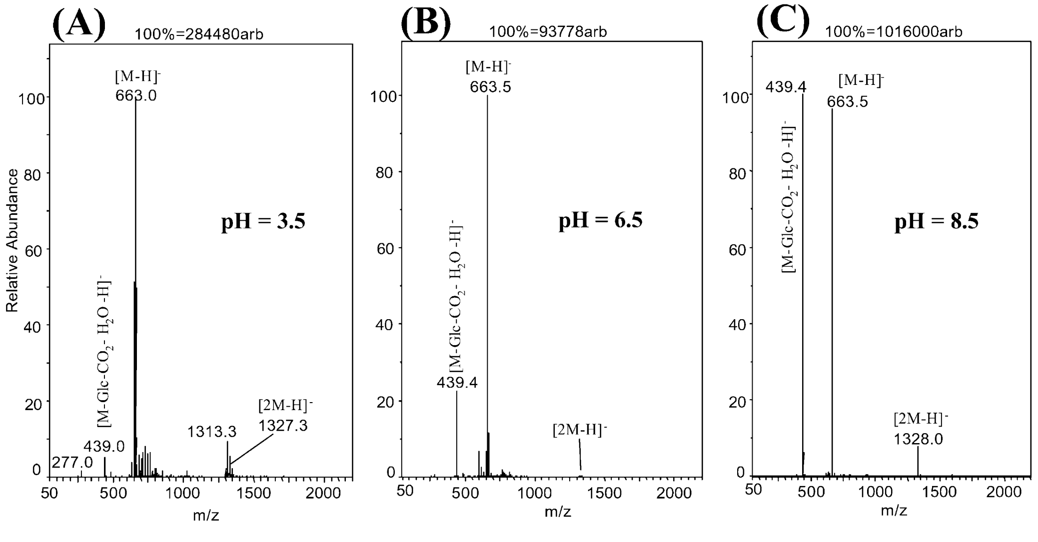


Fig. 3. Base-induced hydrolysis of the sugar conjugate followed by decarboxylation and dehydration of the aglycone, $[M–Glc–CO_2–H_2O–H]^-$, as a function of pH for 3-*O*-β-D-glucopyranosyl medicagenic acid.

Table 1
Effects of mobile phase modifiers on the separataion efficiency of 3-Glc-medicagenic acid[a]

Mobile phase modifier	pH	R_t (min)	$W_{\frac{1}{2}}$ (min)	Mean efficiency $N = 5.54\ (R_t/W_{\frac{1}{2}})^2$	s.d. (RSD) ($n = 3$)
Formic acid	2.9	22.76	0.420	49,200	1459 (3.00%)
Acetic acid	2.9	22.71	0.412	54,482	3805 (6.98%)
Propanoic acid	2.9	22.78	0.402	49,314	4904 (9.94%)
Trifluoroacetic acid[b]	2.9	22.98	0.485	34,321	3497 (10.19%)
MilliQ water	5.7	22.43	0.513	20,083	1173 (5.84%)
8mM Ammonium acetate buffer to pH 7.0 with NH_4OH[c]	7.0	4.28	0.283	1215	133 (10.95)

[a] s.d., standard deviation; RSD, relative standard deviation; N, chromatographic efficiency; R_t, retention time (min); $W_{\frac{1}{2}}$, peak width at half-height (min).

[b] See Burnouf-Radosevich et al., (1986).

[c] See Fuzzati et al. (1999).

An experimental accommodation for both pH conditions would be post column addition of a base to the acidic HPLC eluent at a rate and concentration sufficient to generate a final pH of approximately 8.5. An experiment using flow-injection analyses was designed to simulate the post-column addition of a base to an acidic HPLC eluent. This was performed by titrating a 0.1% acetic acid solution with sufficient NH_4OH to yield a final pH of approximately 8.5. The solution was then used as a mobile phase for flow-injection analysis of soyasaponin I, identical to the conditions used to record the data presented in Fig. 2. Surprisingly, the negative-ion ESI spectra obtained for this solvent system yielded lower ion abundances than those recorded for 0.1% acetic acid only. The lower ion abundance was attributed to the higher ionic strength of the resultant neutralization reaction. It has long been realized that increasing ionic strengths progressively deteriorate ESI instrument response (Smith et al., 1991).

Our results suggest that the best method for profiling complex saponin mixtures from alfalfa and *M. truncuala* is negative-ion HPLC/ESI/MS using a 0.1% acetic acid mobile phase. This decision was based on the higher signal-to-noise for negative-ion ESI and on enhanced chromatographic separation efficiencies of acidic mobile phases. When increased structural information is necessary, data dependent negative-ion HPLC/MS/MS (tandem mass spectrometry) using the same acidic mobile phase or in-source collisional induced dissociation were utilized.

To quantify the performance of our method, a sequential dilution series of soyasaponin I was analyzed by negative-ion HPLC/ESI/MS using a 0.1% acetic acid modifier. This on-column method yielded a limit of detection of less than 1 pmol as illustrated in Fig. 4. Fig. 4 also compares mass selective and UV detection methods. Underivatized saponins lack a strong chromophore; however, the double bond found in the “C” ring does absorb slightly in the UV and can be detected at 206 nm. The data clearly illustrate that ESI/MS detection is more sensitive than UV detection and has the added benefit of selectivity. It is also noted that the 206 nm UV response is strongly dependent on the changing solvent composition in gradient elution HPLC as evidenced by the steep sloping base-line observed in the UV chromatogram. Additional model compounds purified from alfalfa by an independent laboratory (Section 4) were also used to determine HPLC retention times and mass spectral characteristics. These model compounds included 3-Glc-medicagenic acid, 3-Glc-28-Glc-medicagenic acid, Rha-Gal-GalA-soyasapogenol B (soyasaponegenol I) and 3-Ara-Glc-Ara-hederagenin. These retention times and mass spectral characteristics were identical to those listed in Table 2. Repetitive HPLC/MS analyses ($n = 7$) of the same alfalfa extract were performed to quantify the analytical variance of this method of saponin profiling. The average relative standard deviation based on the absolute ion abundance of eleven individual components of various concentrations was determined to be 9.8%.

2.2. *Profiling saponins from M. sativa and M. truncatula*

The optimized, negative-ion HPLC/ESI/MS parameters were then used to profile and compare saponin from *M. sativa* and *M. truncatula* root extracts. The resultant total ion chromatograms are presented in Fig. 5. Identification of individual saponins and a summary of the observed ions are provided in Table 2. Saponins in alfalfa were identified based on HPLC/ESI/MS, HPLC/ESI/MS/MS data, model saponin ESI/MS/MS data and literature references (Bialy et al., 1999). Proposed identifications of saponins in *M. truncatula* were based on HPLC/ESI/MS, HPLC/ESI/MS/MS data, co-migration times with identified alfalfa saponins, and characteristic MS properties observed for alfalfa saponins.

HPLC/ESI/MS profiling of alfalfa roots showed little variance between the cultivars Radius, Kleszczewska (Fig. 5a,b), and Apollo (data not shown); however, a large difference in the number of the saponins was observed between *M. truncatula* and alfalfa. Putative

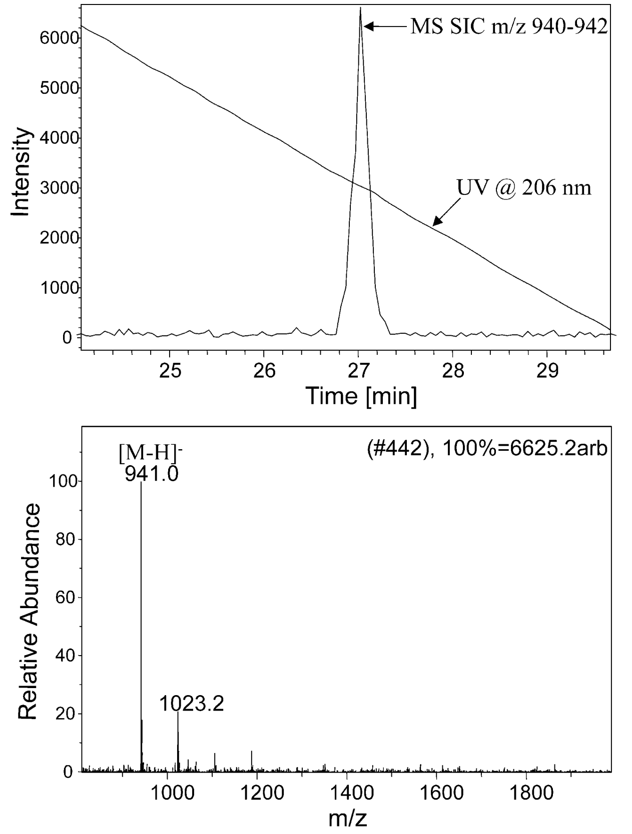


Fig. 4. Analysis of 1.0 pmol Soyasaponin I injected on-column by negative-ion HPLC/ESI/MS and gradient elution with 0.1% aqueous acetic acid and acetonitrile; 20–80% B over 60 min. (A) UV signal recorded at 206 nm and the selected ion chromatogram of *m/z* 940-942 and (B) background subtracted mass spectrum of 1 pmol soyasaponin I.

identifications for the major peaks numbered in Fig. 5 are provided in Table 2. Although some compounds listed in Table 2 could not be resolved in the chromatographic traces they could be easily differentiated using the resultant mass spectral data.

The literature reports 3-Glc-medicagenic acid as a major extractable alfalfa root saponin (Oleszek et al., 1990a,b; Bialy et al., 1999). 3-Glc-medicagenic acid has a molecular weight of 664 Da and was observed immediately and only partially resolved from the base peak; however the base peak (No. 21 in Fig. 5) in all alfalfa profiles yielded a $[M–H]^-$ anion at *m/z* 749. This was confirmed as the molecular anion by the presence of dimer ion, $[2M-H]^-$, at *m/z* 1499. Also present was the characteristic *m/z* 439 anion associated with medicagenic acid conjugates. Additional anions were observed at *m/z* 705 correlating to the loss of 44 and *m/z* 663 correlating to the loss of 86 from the molecular species. These ions are identified as the loss of CO_2 and malonyl group respectively. As a result, we propose the identity of the major extractable saponin in alfalfa to be 3-*O*-β-D-glucopyranosyl-6″-malonyl medicagenic acid. A similar malonated glycoside conjugate was also identified in alfalfa as 3-*O*-β-D-glucopyranosyl-6″-malonyl-28-*O*-β-D-glucopyranosyl medicagenic acid (Peak No. 6). The spectra of the two glycosylated conjugates and their corresponding malonated conjugates are reported in Fig. 6. Although the authors are not aware of previous identification of any malonated glycosides of saponins in alfalfa, the presence of malonated glycosides of flavonoids have been reported for alfalfa supporting the presence of natural product malonyl transferase enzymes in this species (Sumner et al., 1996). The proposed positional configuration of the malonate is based on those associated with flavonoids but is unconfirmed at this point. The presence of malonyl-ginsenosides (acidic saponins) in *Panax ginseng* roots (Fuzzati et al., 1999) and acylated saponins from *Crocosmia* plants (Asada and Furuya, 1996) provide further evidence of the presence of carboxyl glycoside saponin derivatives in plants. The proposed malonated conjugates undergo a shift in retention time when using mobile phases without an acid modifier and monitoring by HPLC/MS. This is illustrated by 3-*O*-β-D-glucopyranosyl-6″-malonyl-28-*O*-β-D-glucopyranosyl medicagenic acid that elutes very close to the void volumne, i.e. R_t equals 2.20 min without acid (pH = 6.9), but 19.35 min with acid (pH = 3.5). This shift in retention time further supports the presence of a carboxy acid that can easily dissociate at neutral pH values. It could also explain why these compounds have not been reported in other HPLC profiling methods that do not use acidified mobile phases (Burnouf-Radosevich and Delfel, 1986; Oleszek et al., 1990a; Bialy et al., 1999).

Mass spectrometry is often incapable of differentiation of isomers, however, our multidimensional approach was able to differentiate selected isomers such as 3-Glc-Glc medicagenic acid from 3-Glc-28-Glc medicagenic acid based on HPLC retention times. Both compounds yield mass spectra with a $[M–H]^-$ anion at *m/z* 825 and the typical *m/z* 439 fragment ion associated with medicagenic acid; however, the 3-Glc-28-Glc-medicagenic acid conjugate elutes much earlier ($R_t = 17.8$ min) than the 3-Glc-Glc-medicagenic acid conjugate ($R_t = 26.4$ min) in reversed phase separations with an acid modifier. The difference in the retention times is based on the ability of the hydrophilic carbohydrates to shield the hydrophobic aglycone from the hydrophobic stationary phase and minimize van der Waals interactions. The 3-Glc-28-Glc medicagenic acid disaccharide has hydrophilic shielding on both ends of the hydrophobic aglycone reducing its interaction with the stationary phase and lowering its retention time. Conversely, the 3-Glc-Glc medicagenic acid conjugate is polarized with the disaccharide forming a hydrophilic end opposite the hydrophobic aglycone that is free to interact with the hydrophobic stationary phase. Similar trends can be observed but are not described in many of

Table 2
Saponins observed by negative-ion HPLC/ESI/MS and HPLC/ESI/MS/MS in *M. sativa* and *M. truncatula*[a]

Peak No.	*M. sativa* Alfalfa	*M. truncatula*	Saponin	R_t (min)	Observed ESI/HPLC/MS	Observed ESI/HPLC/MS/MS ions
1		x	Hex-Hex-Hex-Bayogenin[b]	17.00	1949.2(0.8)$[2M-H]^-$;973.2(13)$[M-H]^-$;811.5(100) $[M-Hex-H]^-$;649.5(14)$[M-Hex-Hex-H]^-$;487.5(10)$[Agly-H]^-$	811.5$[M-Hex-H]^-$;648.3(30)$[M-Hex-Hex-H]^-$;486.5(100) $[Agly-H]^-$
2	x		3-Glc-Glc-Rha-28Glc-Medicagenic acid	17.16	1133.6(100)$[M-H]^-$;971.3.(38)$[M-Glc-H]^-$;811.4(14) $[M-Glc-Glc-H]^-$;649.3(5)$[M-Glc-Glc-Glc-H]^-$;439.4(28) $[Agly-CO_2-H_2O-H]^-$;	1133.8(45)$[M-H]^-$;970.9(100)$[M-Glc-H]^-$
3		x	Rha-Hex-Hex-Hederagenin[b]	17.74	941.4(100)$[M-H]^-$;795.5(33)$[M-Rha-H]^-$; 632.5(33) $[M-Rha-Hex-H]^-$;471.4(12)$[Agly-H]^-$	794.7(100)$[M-Rha-H]^-$;632.5$[M-Rha-Glc-H]^-$;470.6(75) $[Agly-H]^-$
4		x	Hex-Bayogenin[b]	17.98	649.2(100)$[M-H]^-$;487.1(2.5)$[Agly-H]^-$	486.3(100)$[Agly-H]^-$
5	x	x	3-Glc-28-Glc-medicagenic acid	18.66	825.4(100)$[M-H]^-$;663.4(2.5)$[M-Glc-H]^-$; 601.4(3.0) $[M-Glc-CO_2^-H_20-H]^-$;439.3(19)$[Agly-CO_2-H_2O-H]^-$	662.4(5)$[M-Glc-H]^-$;600.2(30)$[M-Glc-CO_2H_2OH]^-$;438.6(100) $[Agly-CO_2-H_2O-H]^-$
6	x	x	3-Glc-malonyl-28-Glc-Medicagenic acid[b]	19.11	1824.8(10)$[2M-H]^-$;911.2(25)$[M-H]^-$;867.5(100) $[M-CO_2\_H]^-$;825.7(10)$[M-malonyl-H]^-$;601.5(10) $[M-malonyl-Glc-CO_2^-H_2O-H]$-439.4(28)$[Agly-CO_2-H_2O-H]^-$	866.8(100)$[M-CO_2-H]$-;600.8(10)$[M-CO_2-malonyl-Glc-CO_2H_2O-H]$-; 438.6(50)$[Agly-CO_2-H_2O-H]^-$
7	x		Ara-Glc-Ara-Hederagenin	19.89	897(100)$[M-H]^-$;765.5(14)$[M-Ara-H]^-$; 603.5(18) $[M-Ara-Glc-H]^-$;471.5(2)$[Agly-H]^-$	764.9(78)[M-Ara-H]-;746.9(60)$[M-Ara-H_2O-H]$-;603.0(100)$[M-Ara-Glc-H]^-$; 585.4(30)$[M-Ara-Glc-H_2O-H]^-$;471.4(10)$[Agly-H]^-$
8		x	Hex-(New Aglycone)[b]	19.95	647.5(100)$[M-H]^-$;485.3(1.0)$[Agly-H]^-$	484.3(100.0)$[Agly-H]^-$
9		x	Rha-Hex-Hex-Soyasapogenol E[b]	20.24	925.5(100)$[M-H]^-$;779.4(25)$[M-Rha-H]^-$; 617.5(23) $[M-Rha-Hex-H]^-$;455.4(5)$[Agly-H]^-$	778.5(80)$[M-Rha-H]^-$;616.4(100)$[M-Rha-Hex-H]^-$;454.6(15)$[Agly-H]^-$
10	x	x	3-Glc-Ara-28-Glc Hederagenin	21.24	1858.2(1)$[2M-H]^-$;927.2(5)$[M-H]^-$;765.3(100) $[M-Glc-H]^-$;603.2(7.5)$[M-Glc-Glc-H]^-$;471.4(4)$[Agly-H]^-$	764.4(100)[M-Glc-H]-; MS/MS of 765 = 602.5(100) [M-Glc-Glc-H]-;471.4(15)[Agly-H]-
11		x	Hex-Hex-Rha Bayogenin[b]	24.30	957.3(100)$[M-H]^-$;811.3(30)$[M-Rha-H]^-$; 649.3(18)$[M-Rha-Hex-H]^-$;487.3(8)$[Agly-H]^-$	810.5(50)$[M-Rha-H]^-$;648.3(45)$[M-Rha-Hex-H]^-$;486.4(100)$[Agly-H]^-$
12		x	Hex-Pen-Soyasapogenol E[b]	25.41	1498.8(0.5)$[2M-H]^-$;749.4(100)$[M-H]^-$; 587.4(5)$[M-Hex-H]^-$;455.4(2.5)$[Agly-H]^-$	586.3(100)$[M-Hex-H]^-$;454.6(8)$[Agly-H]^-$
13		x	Rha-Hex-Hex-Hederagenin[b]	25.89	1885.0(2.5)$[2M-H]^-$;941.5(100)$[M-H]^-$; 795.5(20)$[M-Rha-H]^-$;633.7(10) $[M-Rha-Hex-H]^-$;471.5(1)$[Agly-H]^-$	794.8(100)$[M-Rha-H]^-$;632.7(98)$[M-Rha-Hex-H]^-$;471.1(50)$[Agly-H]^-$
14	x	x	Hex-Hex-Bayogenin[b]	26.85	1624.8(10)$[2M-H]^-$;811.5(100)$[M-H]^-$; 649.5(10)$[M-Hex-H]^-$;487.4(12)$[Agly-H]^-$	648.4(30)[M-Hex-H]-;486.5(100)$[Agly-H]^-$
15	x	x	3-Glc-Glc-Medicagenic acid	26.84	1653.0(35)$[2M-H]^-$;825.5(100)$[M-H]^-$;663.4(25) $[M-Glc-H]^-$;601.5(2.5)$[M-Glc-CO2H20H]^-$; 439.4(28)$[Agly-CO_2-H_2O-H]^-$;	438.5(100)$[Agly-CO_2-H_2O-H]^-$
16	x	x	3-Rha-Gal-Glc-Soyasapongenol B (Soyasapogenol 1)	26.79	1885.1(10)$[2M-H]^-$;941.5(100)$[M-H]^-$	940.8(18)$[M-H]^-$;922.8(30)$[M-H_2O-H]^-$;878.9(27);794.7(12) $[M-Rha-H]^-$; 732.8(40);632.7(4)$[M-Rha-Gal-H]^-$;614.7(38) $[M-Rha-Gal-H_2OH]^-$; 524.8(100);456.9(20 $[Agly-H]^-$
17	x		3-Glc-Ara-28-Glc-Hederagenin	27.46	927.2(100)$[M-H]^-$;765.3(15)$[M-Glc-H]^-$; 603.3(5)$[M-Glc-Glc-H]^-$;471.1(5)$[Agly-H]^-$	764.8(70)[M-Glc-H]-;602.7(100)[M-Glc-Glc-H]-;470.6(18)[Agly-H]-
18		x	Hex-Hex-(New Aglycone)[b]	27.90	1619.8(4)$[2M-H]^-$;809.5$[M-H]^-$; 647.5(2.5)$[M-Hex-H]^-$;485.4(5)$[Agly-H]^-$	646.3(75)$[M-Hex-H]^-$;484.4(100)$[Agly-H]^-$
19		x	Hex-Soyasapogenol B[b]	28.50	1300.1(9)$[2M-H]^-$;649.0(100)$[M-H]^-$	486.5(100)$[Agly-H]^-$
20	x	x	3-Glc-medicagenic acid	28.63	1327.6(4)$[2M-H]^-$;663.4(100)$[M-H]^-$; 439.3(22)$[Agly-CO_2-H_2O-H]^-$	438.4(22)$[Agly-CO_2-H_2O-H]^-$

(*continued on next page*)

Table 2 (*continued*)

Peak No.	*M. sativa* Alfalfa	*M. truncatula*	Saponin	R_t (min)	Observed ESI/HPLC/MS	Observed ESI/HPLC/MS/MS ions
21	x	x	3-Glc-malonyl-Medicageinc acid[b]	28.93	1499.6(10)[2M-H]$^-$;749.2(98)[M-H]$^-$; 705.4(80)[M-CO$_2^-$H]$^-$;633(20)[M-malonyl-H]-; 439.3(100)[Agly-CO$_2$-H$_2$O-H]$^-$	704.7(100)[M-CO$_2$-H]$^-$;438.8(50)[Agly-CO$_2$-H$_2$O-H]$^-$
22	x	x	Rha-Gal-GlcA-Soyasapogenol E	29.46	1881.0(5)[2M-H]$^-$;939.5(100)[M-H]$^-$	938.9(18)[M-H]$^-$;920.9(44)[M-H$_2$O-H]$^-$876.9(38); 794.8(40) [M-Rha-H]-; 731.8(40);632.5(5)[M-Rha-Gal-H]$^-$;612.7(38) [M-Rha-Gal-H$_2$OH]-; 522.6(100);456.9(20)[Agly-H]$^-$
23	x	x	Rha-Hex-Hex-Soyasapogenol E[b]	29.95	1853.0(3)[2M-H]$^-$;925.7(100)[M-H]$^-$; 779.5(20)[M-Rha-H]$^-$;617.7(7)[M-Rha-Glc-H]$^-$	778.5(88)[M-Rha-H]$^-$;616.3(100)[M-Rha-Glc-H]$^-$; 454.5(100)[Agly-H]$^-$
24	x	x	Ara-Hex-Hederagenin	30.64	1532.8(7)[2M-H]$^-$;765.5(100)[M-H]$^-$; 603.5(14)[M-Hex-H]$^-$;471.4(2.5)[Agly-H]$^-$	602.5(100)[M-Hex-H]$^-$;470.8(16)[Agly-H]$^-$
25		x	Hex-Hederagenin[b]	31.20	1268.5(1.5)[2M-H]$^-$;633.5(100)[M-H]$^-$; 471.4(5)[Agly-H]$^-$	470.4(100)[Agly-H]$^-$
26		x	GlcA-Hederagenin[b]	34.42	647.4(100)[M-H]$^-$;471.5(5)[Agly-H]$^-$	470.4(100)[Agly-H]$^-$
27		x	Hex-Hex-Soyasapogeol E[b]	34.76	779.3(100)[M-H]$^-$;617.5(14)[M-Hex-H]$^-$; 455.3(2.5)[Agly-H]$^-$	616.4(100)[M-Hex-H]$^-$;454.5(100)[Agly-H]$^-$
28	x	x	Pen-Hederagenin[b]	35.42	603.5(100)[M-H]$^-$;471.5(5)[Agly-H]$^-$	470.4(100)[Agly-H]$^-$
29		x	Hex-Hederagenin[b]	36.07	1267.5(1.5)[2M-H]$^-$;632.9(100)[M-H]$^-$; 471.4(1.8)[Agly-H]$^-$	471.4(100)[Agly-H]$^-$
30		x	Hex-Soyasapogenol E[b]	40.04	1236.5(.45)[2M-H]$^-$;617.5(100)[M-H]$^-$; 455.5(1)[Agly-H]$^-$	454.6(100)[Agly-H]$^-$

[a] x indicates that the identified saponin was observed; Hex is a hexose such as Glc or Gal; Pen is a pentose such as Xyl or Ara; Glc, β-D-glucopyranosyl; Gal, β-D-galactopyranosyl; GlcA, β-D-glucuronopyranosyl; Rha, α-L-rhamnopyranosyl; Xyl, β-D-xylopyranosyl; Ara, α-L-arabinopyranosyl.

[b] Not previously reported or identified in alfalfa.

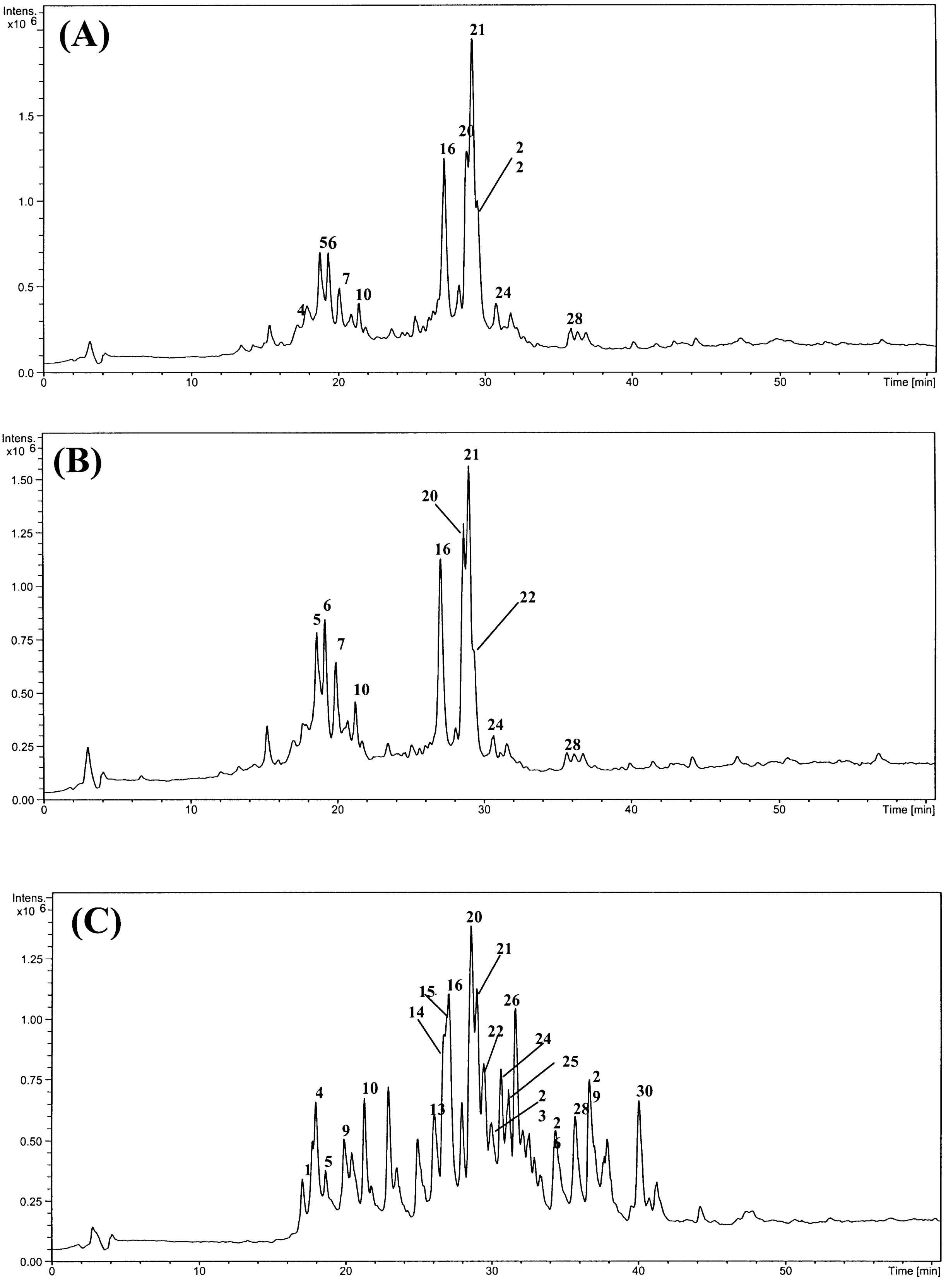


Fig. 5. Total ion chromatograms obtained by negative-ion HPLC/ESI/MS of root extracts of (A) *M. sativa*, cultivar Radius, (B) *M. sativa*, cultivar Kleszczewska, and (C) *M. truncatula*, cultivar Jemalong A17. Separation was achieved using injections of 10 µg total extract, reversed-phase HPLC and gradient elution with 0.1% aqueous acetic acid and acetonitrile (20–80% acetonitrile over 60 min). The peak numbers correspond to saponin identifications presented in Table 2.

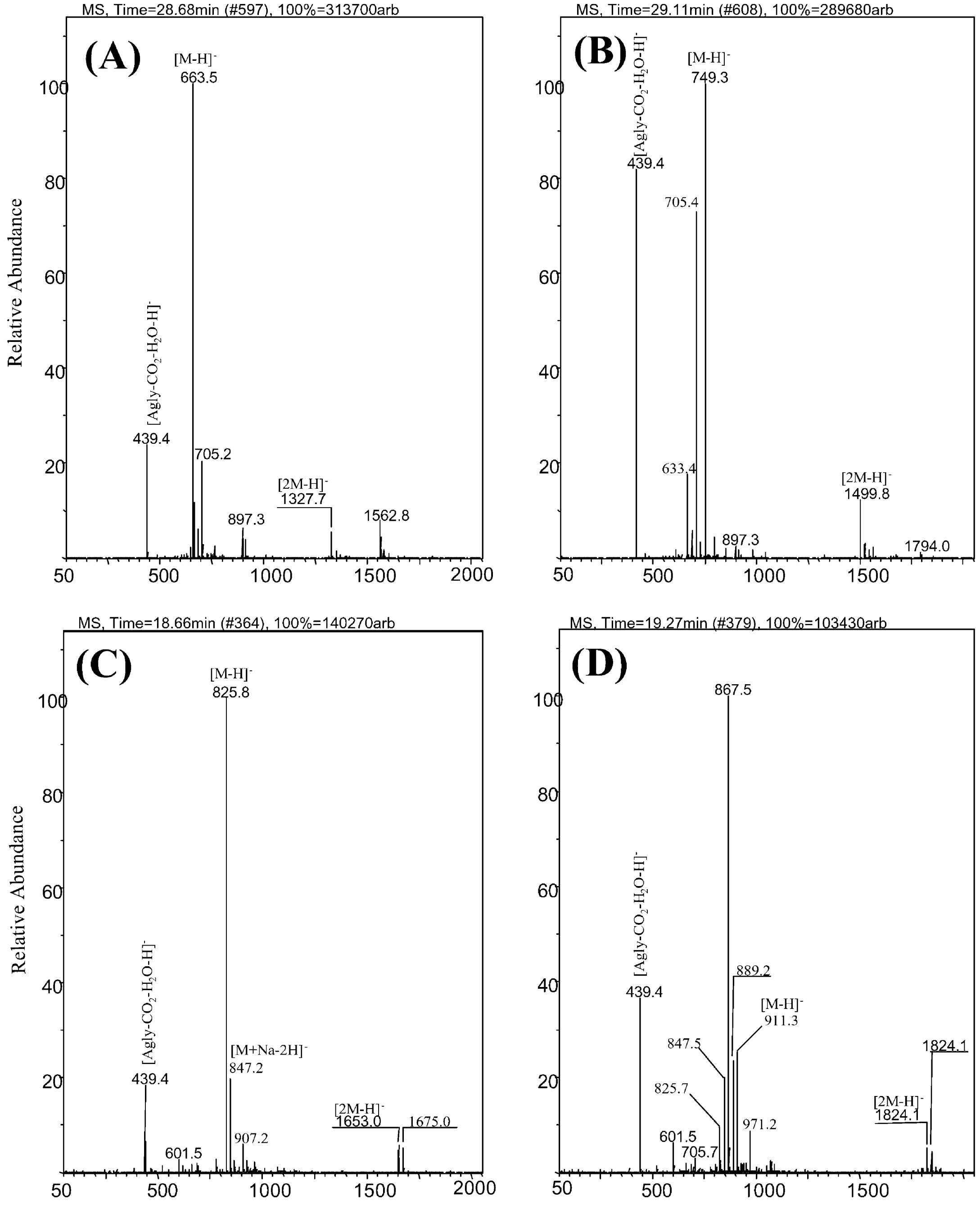


Fig. 6. Negative-ion HPLC/ESI/MS of (A) 3-*O*-β-D-glucopyranosyl medicagenic acid, (B) 3-*O*-β-D-glucopyranosyl-6″-malonyl-medicagenic acid, (C) 3-*O*-β-D-glucopyranosyl-28-*O*-β-D-glucopyranosyl medicagenic acid, and (D) 3-*O*-β-D-glucopyranosyl-6″-malonyl-28-*O*-β-D-glucopyranosyl medicagenic acid.

the literature reports (Domon et al., 1984; Oleszek et al., 1990a; Perret et al, 1999).

To increase the confidence in the putative identifications data dependent tandem mass spectrometry (HPLC/ESI/MS/MS) was performed. This instrumental procedure first isolates molecular ions of interest followed by resonance excitation resulting in collisional activation and dissociation. The resulting fragment ions are directly related to the precursor ion and utilized in structural elucidation. Representative MS/MS spectra are provided in Fig. 7 and all MS/MS results are summarized in Table 2. Distinct ions were generally observed that could be correlated to sequential losses of sugars and the aglycone providing further evidence of structure.

Using all the methods described above, a total of 15 saponins were identified in alfalfa (Table 2). Negative-ion HPLC/ESI/MS and HPLC/ESI/MS/MS, along with HPLC retention times, were used to differentiate and identify twenty-seven independent saponins in *M. truncatula* which are also reported in Table 2. Columns one and two of Table 2 indicate the presence and/or absence of the reported saponins in both species and provides a rapid means of comparison. The authors are not aware of the previous identification of any saponin glycosides in *M. truncatula*; however, a selected list of aglycones has been reported for *M. truncatula* (Jurzysta and Waller, 1996). *M. truncatula* yielded a much more complex mixture of saponins relative to alfalfa. Most of the *M. truncatula* saponins are proposed to be conjugates of medicagenic acid, hederagenin, bayogenin, soyasapogenol B, and soysapongenol E. Most of these aglycones are found in alfalfa however a greater degree of conjugation is

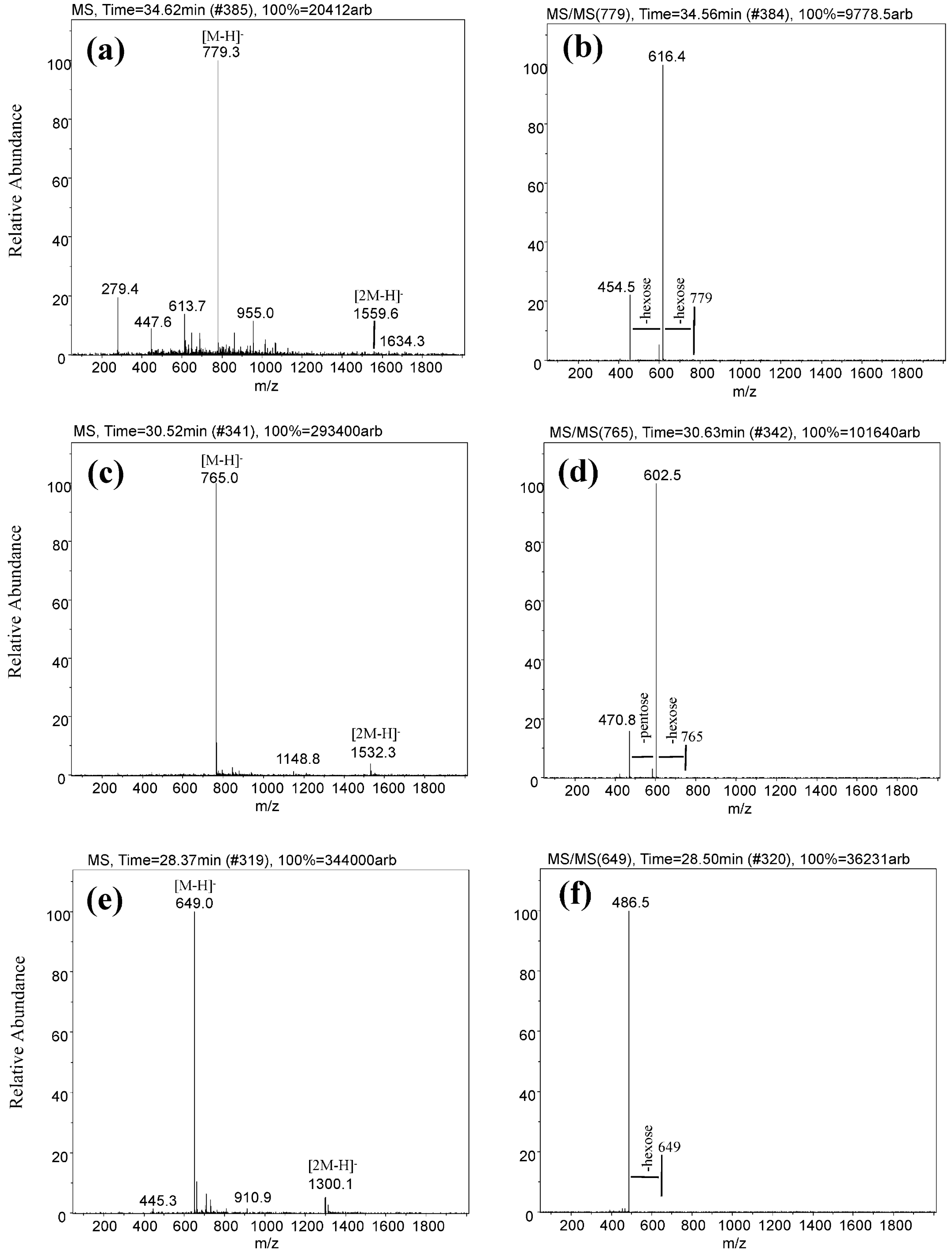


Fig. 7. Example negative-ion HPLC/ESI/MS full scan and tandem mass spectra of several representative saponin aglycone classes observed from *M. truncatula*: (a) MS of Hex-Hex-soyasapongenol E, (b) MS/MS of Hex-Hex-soyasapongenol E, precursor ion is *m*/*z* 779.3, (c) MS of Pen-Hex-hedragenin, (d) MS/MS of Pen-Hex-hedragenin, precursor ion is *m*/*z* 765, (e) MS of Hex-bayogenin, and (f) MS/MS of Hex-bayogenin, precursor ion is *m*/*z* 649.

observed in *M. truncatula*. Example HPLC/ESI/MS and HPLC/ESI/MS/MS mass spectra of each class of the proposed conjugates are provided in Fig. 7.

Many of the compounds were observed as coeluting peaks. Fortunately, MS detection allows for the differentiation and structural characterization of co-eluting compounds (Perret et al., 1999). Many of the *M. truncatula* saponins are reported only as hexoses (pyranosides) or deoxyhexoses as their stereochemistry could not be readily elucidated using mass spectrometry; however we have included these because they could be easily differentiated with our method and to point out an area of needed structural research. The fact that glucose, galactose, galacturonic acid, arbabinose, and xylose are the most common carbohydrates found in association with sapongenins in alfalfa should aid in future identification of these conjugates, however, absolute stereochemical structures will need to be confirmed using NMR.

3. Conclusions

Our results illustrate HPLC/PDA/ESI/MS/MS as a very useful tool in metabolic profiling. We have used this technology not only to compare metabolic profiles of alfalfa and *M. truncatula,* but also to putatively identify a large number of saponins in *M. truncatula*. In addition to the saponins identified, we could detect and differentiate an additional 30 components in both species. Many of these components appear to be saponins, but were not included due to low confidence in their chemical identifications. These additional components add to the range of the reported method and may prove to be important in future studies. The effectiveness of HPLC/PDA/ESI/MS/MS has also been demonstrated in the identification of novel malonated saponins in alfalfa. Malonylation may be important in vacuole storage of saponins or may impart additional biological activity.

Our results also demonstrate that there is a significantly broader range of saponins in *M. truncatula* than in alfalfa. The broader range is primarily due to higher degrees of conjugation in *M. truncatula*. This may have significant implications for storage, roles of saponoins in signaling, and/or plant defense. Furthermore, the greater diversity in conjugation of *M. truncatula* saponins may reflect broader or greater bioactivity, since recent studies of *A. victoriae* saponins suggest that conjugated saponins have greater biological activity than the corresponding aglycones against cancer cells (Haridas et al., 2001).

The above studies provide a foundation for further metabolic profiling of saponins in the context of a broader functional genomics program in *M. truncatula*, a species for which extensive expressed gene sequence information has been reported (Bell et al., 2000). For example, the reported saponin identifications and expressed gene sequence data can be utilized in an integrated functional genomics approach to characterize the biosynthetic pathway of saponins in *M. truncatula* (Suzuki et al., 2001). It is anticipated that this approach will facilitate the identification of structural and regulatory genes associated with complex saponin biosynthesis. Although the present report has focused on saponins, the approach of simultaneous profiling, differentiation, and structural characterization using multidimensional HPLC/PDA/MS/MS can be adopted for other metabolite subclasses (i.e. flavonoids/isoflavonoids) in metabolomic projects.

4. Experimental

4.1. General experimental

HPLC grade acetonitrile was obtained from Burdick and Jackson. HPLC grade methanol was obtained from J.T. Baker (Phillipsburg, NJ). Solid phase extraction (SPE) cartridges (C18, 35 ml, 10 g) were obtained from Waters Corp (Milford, MA). All other chemicals were obtained from Sigma Chemical Co (St. Louis, MO) and used as received.

4.2. Biological materials

Due to the lack of commercially available saponin standards, model compounds from alfalfa (*M. sativa*, Polish variety Kleszczewska) were purchased from W. Oleszek (Department of Biochemistry, Institute of Soil Science and Plant Cultivation, Pulawy, Poland). Model saponins were isolated from alfalfa roots using low pressure Si and RP18 columns and were purified as reported (Bialy et al., 1999). Structures were established by FAB–MS and ^{1}H and ^{13}C NMR.

Saponin extracts for profiling were obtained from *M. truncatula* (Jemalong, cv. A17) and *M. sativa* (cv. Radius, Kleszczewska, and Apollo) roots using a solid phase extraction technique previously described (Oleszek, 1988; Oleseck et al., 1990b). Plants were grown in Turface MVP™ (Profile Products, Buffalo Grove, IL) medium and in a controlled greenhouse environment maintained at an average temperature of 28 °C, 40% humidity, and a daylength of 16 h. Roots from approx. 16–25 plants (~12–32 g dry weight) were removed from the Turface™ medium and allowed to air dry prior to extraction with 80% methanol overnight at ambient temperature. The extracts were concentrated under a nitrogen stream to remove the organic component yielding an aqueous solution. This solution was diluted to yield a final 35% methanol (v/v) solution that was then loaded onto a 35 ml, 10 g, C18 SPE extraction cartridge. The SPE cartridge was then washed with two column volumes each of 100% water and 35% methanol. The saponins were then eluted with two column volumes of 100% methanol. The methanol fraction was taken to dryness using a rotary evaporator, resuspended in methanol to a final concentration of ca. 400 ng/μl and analyzed by gradient elution, reversed-phase HPLC with simultaneous on-line UV and mass selective detection.

4.3. High performance liquid chromatography

HPLC separation was achieved using a reversed-phase, C18, 5 μm, 4.6×250 mm column (J.T. Baker, Phillipsburg, NJ). Samples were eluted with a H_2O:CH_3CN gradient, 80:20 to 20:80 in 60 min, at a flow rate of 0.8 ml/min. Mobile phase modifiers such as 0.1% acetic acid were substituted for the aqueous phase when desired. Post-column addition of ammonium hydroxide to the HPLC column eluent to enhance negative-ion abundances was performed by delivering a concentrated solution via a syringe pump (e.g. 4 μl/min of 30% NH_4OH) to a PEEK tee connector where it was mixed with the HPLC column eluent to yield a final concentration of 0.15% NH_4OH (pH $\sim$ 9.6) at the outlet of the ESI spray nozzle.

4.4. Mass spectrometry

All mass spectra were acquired using a Bruker Esquire ion-trap mass spectrometer equipped with an electrospray ionization source. Both positive and negative-ion mass spectra were acquired. Negative-ion ESI was performed using an ion source voltage of 4.0 kV and a capillary offset voltage of −86.0 V. Nebulization was aided with a coaxial nitrogen sheath gas provided at a pressure of 60 psi. Desolvation was aided using a counter current nitrogen flow set at a pressure at 11 psi and a capillary temperature of 300 °C. Mass spectra were recorded over the range 50–2200 m/z. The Bruker ion-trap mass spectrometer (ITMS) was operated under an ion current control (ICC) of approximately 10,000 with a max aquire time of 200 ms. Longer acquire times and/or ICC values greater than 25,000 often resulted in mass shifts attributed to space-charge effects (March and Todd, 1995). Tandem mass spectra were obtained using both manual and automated MS/MS. Automated MS/MS was performed by isolating the base peak (parent ion) above m/z 400 using an isolation width of 2.0, fragmentation amplitude of 0.6, threshold set at 15,000 and ion charge control on with max acquire time set at 100 ms.

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