Determination of Alfalfa (*Medicago sativa*) Saponins by High-Performance Liquid Chromatography

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Reversed-phase high-performance liquid chromatography (HPLC) has been successfully applied to the determination of saponins in alfalfa root and aerial parts. It was shown that a single extraction of saponins with 30% MeOH followed by SPE C_{18} Sep-Pak cleanup permits 91 and 96% recoveries from roots and aerial parts, respectively. The most abundant saponins in roots were medicagenic acid glycosides substituted at the C3 position with glucuronic acid or glucose and hederagenin glycoside. The aerial parts contained mainly medicagenic acid glycosides substituted with glucuronic acid at C3, zanhic acid tridesmoside, and soyasaponin I. The results of HPLC analyses were compared to the data obtained with the *Trichoderma viride* test. The total content of saponins in roots determined with HPLC was 2.43% of dry matter, whereas the *T. viride* test gave a concentration of 5% DM. For aerial parts these values were 1.49 and 0.95%, respectively.

INTRODUCTION

Alfalfa saponins are secondary plant metabolites found in roots and aerial parts. Their presence in alfalfa has several important implications. High levels of saponins severely restrict the growth of monogastric animals (Cheeke, 1983; Heghest and Linkswiler, 1980), cause the hemolysis of red blood cells, inhibit the growth of some fungi and insects (Assa et al., 1972; Hober, 1972), or harm the development of plants (Oleszek et al., 1992a).

The alfalfa saponins have been identified as mono-, bis-, or tridesmosides of medicagenic acid, hederagenin, zanhic acid, and soyasapogenol B (Timbekova et al., 1989; Massiot et al., 1988a,b, 1991; Oleszek et al., 1990a, 1992b). Among them, medicagenic acid and hederagenin glycosides have been recognized as the "biologically active" compounds and they were determined with biological tests. The most often used tests are based on inhibition of the growth of *Trichoderma viride* (Zimmer et al., 1967) and plant seedlings (Pedersen, 1975) or on the hemolytic activity (Jurzysta, 1979). However, the results obtained by all of these tests are strongly influenced by both the aglycon and the carbohydrate composition of the saponin molecule (Oleszek, 1990; Oleszek et al., 1990a). Thus, the determination of total saponin content with these biological procedures neither provides the content of individual compounds nor gives any information on their composition. It is highly probable that the relative quantities of particular glycosides may vary under different environmental conditions and may depend on the alfalfa variety. Observed changes in total saponin content under these circumstances (Hanson et al., 1963; Guenzi et al., 1964) may simply arise from the variation in the content of most active compounds. Moreover, glycosides of zanhic acid and soyasapogenol B may show negligible response to biological tests, but their presence in alfalfa cannot be neglected. Therefore, more selective analytical methods are needed to obtain reliable results.

In this paper, we present the determination of individual saponins in alfalfa roots and aerial parts by high-performance liquid chromatography.

MATERIALS AND METHODS

Plant Material. Alfalfa (*Medicago sativa* var. Boja) was grown on the Experimental Farm at Pulawy, Poland. The aerial parts (leaves and stems) and roots were collected in June 1991 (first cut) from a 1-year old alfalfa stand. Samples were air-dried and finely powdered prior to extraction.

Chemicals. Acetonitrile and methanol were of HPLC grade (Baker, Deventer, Holland); bromophenacyl bromide and 18-crown-6 were from Sigma (St. Louis, MO).

HPLC Analysis. *Extraction.* For a typical extraction, 1 g of root or aerial part sample was refluxed for 1.5 h with 100 mL of aqueous 30% MeOH. The extract was centrifuged, and the volume of supernatant was made up to 100 mL with 30% MeOH (first extraction). The residue was extracted two more times by using the same procedure as for the first extraction (second and third extractions).

Purification. Ten milliliters of each sample was passed through a C_{18} Sep-Pak cartridge (Waters Associates) preconditioned with 5 mL of 30% MeOH. The cartridge was washed with 5 mL of 30% MeOH and saponins were removed with 5 mL of HPLC grade MeOH.

Derivatization for HPLC. After evaporation of the solvent, saponins were derivatized with 4-bromophenacyl bromide according to a previously described procedure (Oleszek et al., 1990b).

For the determination of zanhic acid tridesmoside a procedure modified from that of Nowacka and Oleszek (1992) was used. For that, 10 mL of the extract was treated with 1 mL of saturated lead acetate solution in H_2O and the resulting precipitate was centrifuged at 3000*g*. The supernatant was passed through a C_{18} Sep-Pak cartridge and purified as above. The eluted saponin was subsequently derivatized for HPLC after alkaline hydrolysis.

Identification and Quantitation. The saponins derivatized with 4-bromophenacyl bromide were analyzed by an HPLC system (Knauer, Germany), equipped with a computer system to monitor chromatographic parameters and to process the data and a variable-wavelength UV detector operating at 260 nm. Separations were performed on a 5-μm (250 × 4.6 mm i.d.) Eurospher 80 C_{18} column (Saulentechnik, Germany). Chromatographic runs were carried out using a mobile phase (ACN/H_2O) gradient as previously described (Nowacka and Oleszek, 1992). Three independent chromatographic runs were performed for each extract, and saponins were identified by comparing their retention times with those of authentic standards (Oleszek et al., 1990a, 1992b). Quantitation was based on external standardization by employing calibration curves in the range 0.25–2 mg/mL of the reference compounds.

Fungal Bioassay. The fungus *T. viride* method was employed for the estimation of total saponin content of each sample (Oleszek et al., 1992a).

Statistical Analysis. Each sample was submitted to five replicate analyses, and results were subjected to ANOVA test.

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Table 1. Saponin Content of Alfalfa Aerial Parts[a]

compound	saponin content (% of dry matter ± SD)			
	I extract	II extract	III extract	total
3GlcA,28AraRhaXyl Ma	0.571 ± 0.032	traces	0.0	0.571
3GlcA,28AraRha Ma	0.104 ± 0.006	traces	0.0	0.104
soyasaponin I	0.197 ± 0.043	0.063 ± 0.019	0.0	0.260
unidentified 1, 2, 3, 7	0.290 ± 0.018	0.0	0.0	0.290
3GlcGlcGlc,23Ara, 28AraRhaXylApi Za	0.300 ± 0.020	0.0	0.0	0.300
total of extracts	1.462	0.063	0.0	1.525
(extr eff % of total)	(96)	(4)		
T. viride test	0.95 ± 0.05	na	na	0.95

[a] Ma, medicagenic acid; Za, zanhic acid; traces, saponin content below 0.02% in dry matter; na, not active.

Table 2. Saponin Content of Alfalfa Roots[a]

compound	saponin content (% of dry matter ± SD)			
	I extract	II extract	III extract	total
3Glc,28AraRhaXyl Ma	0.368 ± 0.050	0.028 ± 0.004	0.0	0.397
3Glc,28Glc Ma	0.342 ± 0.040	0.046 ± 0.006	0.0	0.388
unidentified 3, 4, 5, 6	0.451 ± 0.040	0.0	0.0	0.451
3GlcA,28AraRhaXyl Ma	0.742 ± 0.042	0.022 ± 0.001	0.0	0.764
3AraGlcAra Hed	0.108 ± 0.005	traces	0.0	0.108
soyasaponin I	0.053 ± 0.005	traces	0.0	0.053
3Glc Ma	0.125 ± 0.009	0.081 ± 0.025	0.044 ± 0.01	0.250
total of extracts	2.189	0.177	0.044	2.410
(extr eff % of total)	(91)	(7)	(2)	
T. viride test	5.0 ± 0.05	na	na	5.0

[a] Ma, medicagenic acid; Hed, hederagenin; traces, saponin content below 0.02% in dry matter; na, not active.

RESULTS AND DISCUSSION

The HPLC separation of individual saponin standards derivatized with 4-bromophenacyl bromide was successfully performed (Oleszek et al., 1990b), suggesting this technique to be a powerful tool for determination of these compounds in plant material. However, to use this procedure for analysis of alfalfa samples, an extraction and cleanup procedure had to be developed. Livingston et al. (1977) showed that by using *T. viride* tests saponins can be readily extracted from alfalfa meals by autoclaving for 0.5 h, but in some cases the extraction with methanol/water extractants gave actually better results. In the present study, several attempts with different MeOH/H_2O mixtures led us to select 30% aqueous MeOH solution as an optimal solvent for alfalfa samples. The efficiency of this extractant was evaluated by HPLC analysis of saponin content in three consecutive extracts of the same sample. The data of this trial are given in Tables 1 and 2. For alfalfa aerial parts, the first extraction removed 96% of saponins, and in the second extraction only a small amount of soyasaponin I (4% of total) was found. The third extraction contained no saponins. In the root samples these values were slightly lower; saponin removal was 91, 7, and 2% of total for the first, second, and third extractions, respectively. Thus, for routine analysis it would be reasonable to use a single extraction with 30% MeOH for aerial parts and duplicate extraction for the roots. The extraction and purification procedure has been checked for recovery efficiency with spiked 3GlcA,-28AraRhaXyl medicagenic acid standard. Recoveries for three trials ranged between 90 and 92%.

The use of 30% MeOH as the extractant was very convenient also from the cleanup procedure point of view. As was shown previously (Oleszek, 1988), the alfalfa saponins retained on the C_{18} Sep-Pak could not be removed with eluant containing less than 40% MeOH. Saponin extract can thus be safely cleaned up on the Sep-Pak cartridge with 30% MeOH without any danger of losing the compounds under investigation. At the same time some components of the extract matrix such as carbohydrates, simple phenolics, and some flavonoid glycosides can be effectively removed. By this procedure, saponins washed out from the Sep-Pak with 100% MeOH are in a sufficiently purified form to be derivatized for HPLC.

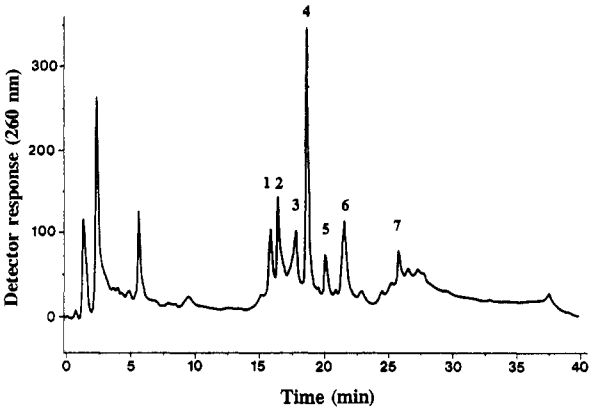

Figure 1. Chromatogram of analytical HPLC of saponins from alfalfa aerial parts: (peaks 1, 2, 3, 7) not identified; (peak 4) 3GlcA,28AraRhaXyl Ma; (peak 5) 3GlcA,28AraRha Ma; (peak 6) soyasaponin I. For separation conditions, see HPLC Analysis.

The method reproducibility was satisfactory. Relative standard deviation (RSD) for saponins from aerial parts was 6–7% (n = 5), whereas for the roots RSD was 9-11% (n = 5). RSD for consecutive injection of the same sample was 2% (n = 3) and for derivatization 5% (n = 3). These deviations, which are relatively high, seem to reflect the problem of low homogeneity of plant material. Pedersen (1975) reported that in alfalfa roots most of the saponins are concentrated in the cortex and not in the stele; similarly in aerial parts, leaves were much richer in saponins than stems.

Chromatograms of alfalfa aerial part and root saponins distinctly differed (Figures 1 and 2). In the chromatogram of aerial parts some peaks with retention times of 1–6 min can be seen, whereas they were not present in the chromatogram of the roots. These peaks were not identified. The other peaks in the chromatogram corresponded

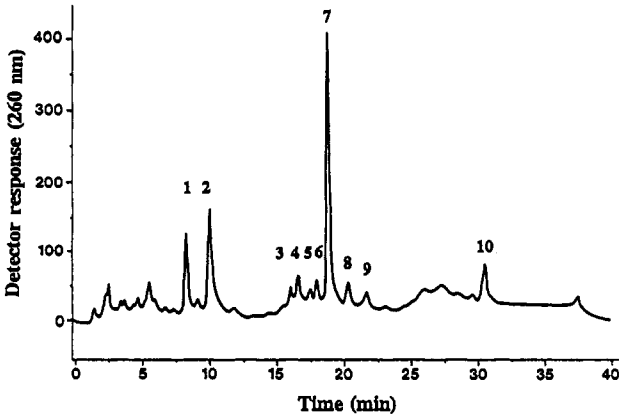

Figure 2. Chromatogram of analytical HPLC of saponins from alfalfa roots: (peak 1) 3Glc,28AraRhaXyl Ma; (peak 2) 3Glc,-28Glc Ma; (peaks 3, 4, 5, 6) not identified; (peak 7) 3GlcA,-28AraRhaXyl Ma; (peak 8) 3AraGlcAra hederagenin; (peak 9) soyasaponin I; (peak 10) 3Glc Ma.

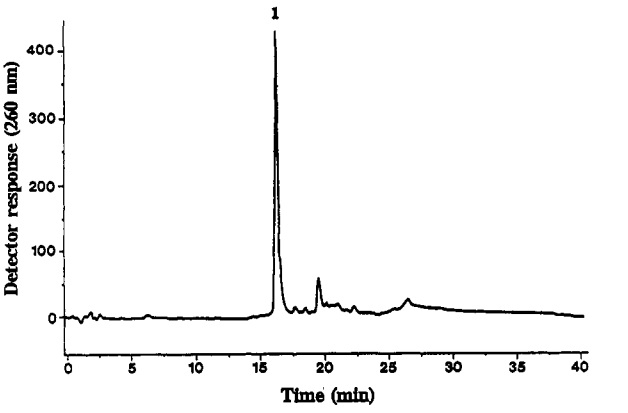

Figure 3. Chromatogram of analytical HPLC of prosapogenin obtained from alkaline hydrolysis of zanhic acid tridesmoside: (peak 1) 3GlcGlcGlc zanhic acid.

to saponins, and those for which standards were available were identified.

The aerial parts of alfalfa contained seven compounds [the structures of compounds represented by peaks 1–3 and 7 (Figure 1) are not known and their total amount was determined in relation to saponin 4], and two of them, i.e., 3GlcA,28AraRhaXyl medicagenic acid and zanhic acid tridesmoside, made up 60% of total saponins. The zanhic acid tridesmoside could not be determined together with other saponins in one HPLC run. This compound has no free carboxylic group and cannot be readily derivatized with 4-bromophenacyl bromide. In our previous paper (Nowacka and Oleszek, 1992) zanhic acid tridesmoside had to be alkaline-hydrolyzed prior to derivatization, and in fact the generated prosapogenin with deblocked COOH groups was determined. But alkaline hydrolysis of the saponin mixture generated also a number of prosapogenins derived from medicagenic acid bisdesmosides. There was a danger, though, that one of these medicagenic acid prosapogenins may overlap the one derived from zanhic acid tridesmoside. To overcome overlapping, in the present work saponin extract prior to alkaline hydrolysis was treated with lead acetate. This treatment precipitated all saponins having a free carboxylic group. When the precipitate was centrifuged, the remaining supernatant contained only zanhic acid tridesmoside, which was further purified on C_{18} solid-phase support. Alkaline hydrolysis of this purified saponin followed by derivatization gave an HPLC chromatogram with one dominant peak (Figure 3) corresponding to 3GlcGlcGlc zanhic acid (Nowacka and Oleszek, 1992).

In alfalfa roots (Figure 2) 10 compounds were identified. They were glycosides of medicagenic acid, hederagenin, and soyasapogenol B. It should be stressed, however, that care has to be taken in identifying compounds according to their retention times. In the present work 3GlcA,-28AraRha medicagenic acid (peak 5, Figure 1) in tops had an R_t identical to that of 3AraGlcAra hederagenin (peak 8, Figure 2) in alfalfa roots. Fortunately, 3GlcA,28AraRha medicagenic acid was not identified in alfalfa roots and hederagenin glycoside was not found in tops, and thus they can be safely determined with HPLC.

Similar to the aerial parts, in roots 3GlcA,28AraRhaXyl medicagenic acid (peak 7, Figure 2) was the most abundant compound (38% of total). Contrary to the aerial parts, roots contained also a considerable amount of medicagenic acid glycosides having glucose at the C3 position (compounds **1**, **2**, and **10**). The presence of compound **1** in aerial parts was previously reported (Massiot et al., 1991; Oleszek et al., 1992b), but its concentration was under the detection limit of the present procedure. Saponins **2** and **11** could only be found in alfalfa roots. Below the detection limit was also the concentration of other saponins which in the number of 20–30 can be distinguished by two-dimensional thin-layer chromatography (Oleszek and Jurzysta, 1986). Recognition of individual saponins on TLC is based on color development with a reagent, but the color intensity of spots corresponding to particular compounds does not necessarily reflect their real concentrations. From our own experience it is known that soyasaponin I can be easily detected on TLC even at relatively low concentration, whereas zanhic acid glycosides give very poor intensity even at quite high amounts. The present HPLC procedure makes it possible to evaluate the real quantity of individual compounds.

As seen from the presented chromatograms (Figures 1 and 2), there are only a few compounds in aerial parts and some more in roots that define the biological activity of alfalfa saponin mixture. The majority of compounds seen on TLC do not seem to play any substantial role in this respect. It must be, however, stressed that the presented chromatograms reflect saponin composition in one alfalfa variety grown under certain environmental conditions. It is highly probable that variety and environment can significantly change saponin composition, as was shown in previous studies revealing variations in saponin content among alfalfa varieties and stages of development (Guenzi et al., 1964; Hanson et al., 1963). It was impossible, however, to relate these variations to the total saponin content or to the structural changes of saponins, since all previous data were based mostly on the *T. viride* test.

The total saponin content in alfalfa roots determined with the HPLC method was 2.4% of dry matter (DM). This result corresponded to the upper level of the concentration range (1.21–2.19% DM) reported by Shany et al. (1970) as based on extraction efficiency, but it was higher than the value 0.96% DM found by Tencer et al. (1972) with the titrimetric method. The total amount of root saponins determined with the *T. viride* test was 5% of dry matter, which is in good agreement with previous estimations performed with fungal and hemolytic biotests (Oleszek and Jurzysta, 1986) and should reflect the content of the biologically active fraction. Thus, application of *T. viride* test in the analysis of alfalfa roots seems to give an overestimation of the saponin concentration, probably due to the exceptional activity of 3Glc Ma against the fungus. As previously reported (Oleszek et al., 1990a), the IA_{50} value for 3Glc Ma was 0.16 mg/100 mL of growth medium, whereas for the dominant saponin 3GlcA,28AraRhaXyl Ma this value was 4.75 mg. Therefore, to elicit comparable

inhibitory effects, the latter compound must be in concentration 30 times higher than that for 3Glc Ma; for other saponins present in the roots these concentrations should be 10–20 times higher. Hence, even small variations of 3Glc Ma may dramatically change the final result.

In the aerial parts the total amount of saponins determined by the *T. viride* test was 0.95% of dry matter, and this value strictly corresponded to the content of 0.94% DM of the biologically active fraction, i.e., total saponins excluding zanhic acid tridesmoside and soyasaponin I, which show no activity against *T. viride* (Oleszek et al., 1990a, 1992b). This value correlated with the data reported by Jurzysta (1982) obtained for a number of alfalfa varieties. In this biologically active fraction 3GlcA,-28AraRhaXyl Ma predominated (60%), and thus, the chromatogram presented (Figure 1) is similar to the one obtained previously for the mixture of saponins used for standard curve preparation for the *T. viride* test (Oleszek, 1991). This resemblance verifies agreement between results of bioassay and HPLC. The advantage of the HPLC procedure over the *T. viride* test is in the possibility of determining biologically active saponins in addition to zanhic acid tridesmoside and soyasaponin I, which cannot be determined with a biological method. It seems that zanhic acid glycosides may prove to be very important antinutritional factors due to their bitterness, throat irritating activity, and ability to change small intestine permeability. The influence of zanhic acid tridesmoside on transmural potential difference in mammalian small intestine was the highest not only of all alfalfa saponins but also of any other plant glycosides tested (Gee et al., 1989; Oleszek et al., 1994). Hence, zanhic acid glycosides cannot be neglected when the antinutritional function of alfalfa saponins is to be considered. The presented HPLC procedure gives great possibilities for a precise look into saponin composition and concentration in plant material.

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