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SAPONINS OF TWO ALFALFA CULTIVARS

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Key Word Index—*Medicago sativa*; Leguminosae; alfalfa; saponins; pentacyclic triterpenoid sapogenins; monosaccharides; structural analysis.

Abstract—A saponin mixture was separated from the forage of DuPuits and Lahontan cultivars of *Medicago sativa* and found to contain about 30 saponins. Glucose, galactose, xylose, arabinose and rhamnose, were the principal sugars; the sapogenins comprised soyasapogenols A and B, lucernic acid, medicagenic acid together with four unidentified but related triterpenoids. Medicagenic acid was the predominant sapogenin of the DuPuits cultivar, whereas soyasapogenol A was prominent in Lahontan saponins. Galactose was found in the saponins of monocarboxylic or nonacidic sapogenins but was absent in those containing dicarboxylic sapogenins, such as medicagenic acid.

INTRODUCTION

IN A NATIONWIDE variability study, 16% of the variation in total saponin concentration of alfalfa forage was attributed to differences among cultivars.[1] Lahontan had the least saponin. A relatively high estimate of heritability of total saponin obtained from this study stimulated selection experiments designed to reduce saponin in each of six cultivars.[2]

Alfalfa meals made from the low saponin strains were superior to those from high-saponin strains in tests for weight gain of chicks, egg production of laying hens and weight gain of rats.[3,4] The saponin fractions of alfalfa have been considered as the possible cause for differences in feeding value and in the resistance to some plant pathogens.[5–7] DuPuits alfalfa saponins showed a distinctly higher inhibition than Lahontan of the growth of the *Trichoderma* species in bioassays.[7] Even though the DuPuits cultivar contained about twice as much saponin as Lahontan alfalfa, this difference could not explain the whole range of differences between the alfalfa cultivars. Qualitative differences in the composition of the saponins were thought to account for the biological characteristics. Although, in

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previous studies,[8] crude mixtures of alfalfa saponins have been subjected to qualitative analysis, there has not, to our knowledge, been a comparison of defined saponin fractions from different alfalfa cultivars. We have summarized some of the differences in saponin characteristics of DuPuits and Lahontan for a symposium on Anti-Quality Components of Forages.[9] In the present study, we provide more detailed information on the chemistry of the saponins of these two alfalfa cultivars.

TABLE 1. TLC RESOLUTION OF DUPUITS AND LAHONTAN ALFALFA SAPONIN MIXTURES BY TWO CONSECUTIVE SOLVENT DEVELOPMENTS

Designation of DuPuits saponin	R_f values (1)*	(2)*	Designation of Lahontan saponin	R_f values (1)*	(2)*
1 A	0·26	0·33	1 A	0·25	0·33
2 B	0·30	0·28	2 B	0·30	0·27
			3		0·30
3		0·29	4		0·26
4		0·36	5 C	0·36	0·33
5		0·40	6		0·38
6 C	0·37	0·45	7		0·42
7		0·50			
8		0·56			
9		0·27	8		0·25
10		0·33	9		0·32
11		0·39	10 D	0·40	0·36
12		0·43	11		0·39
13 D	0·41	0·47	12		0·45
14		0·51			
15		0·56			
16		0·59			
17		0·28	13		0·29
18		0·32	14		0·33
19		0·37	15		0·37
20		0·40	16		0·46
21		0·43	17		0·50
22 E	0·49	0·47	18 E	0·49	0·56
23		0·51	19		0·58
24		0·55	20		0·60
25		0·58			
26		0·62			
27		0·33	21		0·33
28		0·37	22		0·37
29		0·40	23 F	0·60	0·43
30 F	0·60	0·51	24		0·48
31		0·55	25		0·54
32		0·61	26		0·58
33		0·64	27		0·62

* Solvent system (1), BuOH–HOAc–H_2O (4:1:2); solvent system (2), $CHCl_3$–MeOH–H_2O (65:38:10).

[8] BIRK, Y. (1969) *Toxic Constituents of Plant Food Stuffs*, p. 169, Academic Press, New York. See also SHANY, S., GESTETNER, B., BIRK, Y. and BONDI, A. (1972) *Israel J. Chem.* **10**, 881.

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RESULTS AND DISCUSSION

Saponins were isolated by alcohol extractions of whole plant alfalfa meal of DuPuits and Lahontan cultivars followed by butanol fractionation.[10] The saponin obtained proved to be a complex mixture, which on initial TLC consisted of six major zones. These were designated alphabetically in order of increasing R_f values (Table 1). For further resolution, the saponins contained in each of these zones were isolated and subjected to TLC, using a double development technique. The results indicated the presence of a large number of saponins and the R_f values for the DuPuits and Lahontan saponins, using the neutral and acid solvent systems, are shown in Table 1. A total of 33 saponins was detected in DuPuits while Lahontan appeared to contain 27 different saponins.

Although the TLC procedure was a powerful tool for the resolution of alfalfa saponins it was not applicable on a preparative scale. For preparative purposes, the initial saponin mixture was resolved by double development in butanol–acetic acid–water which separated 10 relatively well-defined saponin fractions for each cultivar. By this procedure, fractions A and D in DuPuits saponin (Table 1) were unchanged, B, C, E and F each now gave two components designated B_1, B_2 etc. In the Lahontan saponins, fractions A and C (Table 1) were unchanged, B, D, E and F each yielded two components designated as above. The corresponding R_f values for the zones were: Lahontan—A (0·31), B_1 (0·37), B_2 (0·40), C (0·43), D_1 (0·49), D_2 (0·52), E_1 (0·55), E_2 (0·60), F_1 (0·63), F_2 (0·67); DuPuits—A (0·31), B_1 (0·37), B_2 (0·40), C_1 (0·43), C_2 (0·46), D (0·51), E_1 (0·55), E_2 (0·60), F_1 (0·63), F_2 (0·67). This method enabled the isolation of 20 (10 mg) saponin fractions which were then hydrolyzed. Except for the DuPuits and Lahontan fractions A, this treatment yielded monosaccharides and sapogenins from all fractions without extensive decomposition.

The sapogenins were identified by high-resolution MS of the corresponding acetates and methyl esters. The MS fragmentation of all sapogenin derivatives followed the same general pattern. Compounds with three or four substituents showed intense peaks for M^+, whereas sapogenin derivatives with five or more functional groups gave rise to relatively small signals for M^+ but showed intense peaks at M^+-60. The presence of each hydroxyl group was indicated by the loss of 60 a.m.u. (HOAc). Fragments which had already lost 2 or more molecules of HOAc further fragmented by eliminating 42 mass units (CH_2CO). The presence of carboxyl substituents was indicated by the elimination of 59 a.m.u. (MeOCO). This process was most prominent only after the previous loss of at least two hydroxyl functions as HOAc. The elimination of the angular substituent at C-28 gave rise to more abundant fragments, in particular those related to (**9**) or (**10**). These fragments which resulted from a characteristic retro-Diels-Alder fragmentation[11] of the pentacyclic triterpenoid parent structure were observed for all sapogenins listed in Table 2. The fragmentation of (**9**) [or (**10**) respectively] also followed the rule that any acetoxy groups present were lost as HOAc before the cleavage of carboxymethyl residues occurred to any great extent. On the basis of these observations the four known sapogenins (**1**), (**2**), (**5**) and (**6**) could clearly be identified even in mixtures with unknown constituents. Assuming that the unknown compounds were also related to pentacyclic triterpenoids the application of the same rules facilitated the tentative assignment of a few structural features to the unknown constituents (**2**), (**4**), (**7**) and (**8**), as outlined in Table 2. In particular the evaluation of the fragmentation of (**9**) or (**10**) in the MS contributed to the assignment of substituents in ring

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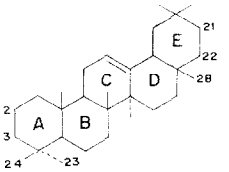
system D or E. All suggested structures were also supported by M^+ ions and elemental formulae.

TABLE 2. SAPOGENINS OF DUPUITS AND LAHONTAN ALFALFA

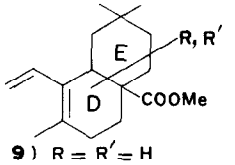


Aglycone designation	Identification	Number of substituents OH	COOH	Structural features	% Total saponin DuPuits	Lahontan
(1)	Medicagenic acid	2	2	2,3-OH 23,28-COOH	40	5
(2)	Unknown	3	2	OH in ring D or E, 28-COOH	15	15
(3)	Soyasapogenol A	4	—	3,21,22,23-OH	10	35
(4)	Unknown	3	1	OH in ring D or E, 28-COOH	10	15
(5)	Lucernic acid	3	1	Lactone	5	10
(6)	Soyasapogenol B	3	—	3, 21, 23-OH	10	10
(7)	Unknown	4		Δ and 2 OH in ring D or E	5	5
(8)	Unknown	5	1	Two OH in ring D or E, 28-COOH	<5	<5

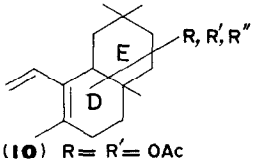
The main fragments in the MS of sapogenin fractions that contained the aglycone (**2**) were observed at mass numbers, (672), 612, 580, 570, 552, 493, 443, 260, 201. There was initially some ambiguity in the structural assignment of this aglycone. The problem involved the question of whether the small peak observed at *m/e* 672 was the M^+, in which case the molecule would contain 3 hydroxyl and 2 carboxyl functions. The intense peak at *m/e* 612, however, could be due to the elimination of HOAc in the mass spectrometer, a commonly observed process, or by elimination of a hydroxyl function under the hydrolysis conditions. Alternatively, the sapogenin fraction could essentially consist of an aglycone which incorporated an additional double bond in rigs D and E. In this case the intense fragment at *m/e* 612 would be the M^+. The following evidence indicated that aglycone (**2**) in fact contained 3 hydroxyl functions. Application of a graded hydrolysis to saponins incorporating (**2**) led to the isolation of a monoglucoside of (**2**), the MS of which showed a small peak at *m/e* 900 (M^+-60), intense peaks at *m/e* 858 (M^+-102), 815 (M^+-145), and 798 (M^+-162). This observation agrees with the usual behaviour of peracetylated glycosides, which do not show an appreciable peak for M^+. These data can be interpreted in favor of a medicagenic acid or an isomer thereof, with one additional hydroxyl substituent in rings D or E, which may form the linkage to the carbohydrate chain, with glucose as the first sugar molecule.

Saponins containing medicagenic acid as the aglycone, on graded hydrolysis also gave rise to monoglucosides. As anticipated, the MS of the methylated and acetylated compound showed corresponding peaks at *m/e* 842 (M^+-60), and 800 (M^+-102). Assuming a basic similarity in the fragmentation pattern of the monoglucosides of (**1**) and (**2**) these findings confirm the structure of the sapogenin (**2**) as having one additional OH group in ring D or E, since the highest detectable peak in the MS of the derivatized monoglucoside of (**2**) was 58 a.m.u. (one acetoxy group) higher than the corresponding derivative of (**1**).

A few structural features could be assigned to the sapogenin (**4**), which has not been previously found in alfalfa. MW (*m/e* 628) and MS fragmentation pattern of the acetylated and methylated aglycone suggested that the material contained three hydroxyl substituents, with one of them located in ring D or E and a carboxyl group at C-28. The corresponding fragments in the MS of the acetylated and methylated (**4**) appeared at *m/e* 628 (M^+), 568, 508, 449, 389, 320 (**9**b), 260 and 201. Even more substituted sapogenins (**7** and **8**) were detected and partially analyzed by MS using fragments (**9**c) and (**10**), the M^+ and elemental formulae.



(**9**) R = R' = H
(**9**a) R = Δ, R' = H
(**9**b) R = OAc, R' = H
(**9**c) R = OAc, R' = OAc



(**10**) R = R' = OAc
(**10**a) R = R' = R'' = OAc

It is conceivable that (**7**) was actually an artifact formed during hydrolysis, by elimination of one OH from a sapogenin containing 3 OH groups in ring D or E. The existence of such an aglycone, with a total of 5 OH substituents, was indeed suggested by the observation of a weak peak of the acetylated compound at *m/e* 700. It was, however, not corroborated by a significant fragment (**10**a) (*m/e* 392). There was also some evidence of the presence of aglycones with more than five substituents and those with less than three. However, these constituents were present only in small concentrations.

In addition to high resolution MS, sapogenin fractions obtained by acid hydrolysis were also subjected to TLC analysis which confirmed that some of the material consisted of several aglycones. Soyasapogenols A and B as well as medicagenic and lucernic acids were identified by comparison with authentic compounds. All aglycones gave characteristic colors. To determine the approximate percentages of individual sapogenins in the two alfalfa cultivars the total saponin mixtures were hydrolyzed and the resulting sapogenin fractions resolved on TLC. The distribution of the aglycones was determined by estimation of color intensities. With the exception of (**4**), all unknown sapogenins showed an approximately equal distribution between the two cultivars. The identity of this sapogenin was concluded from its similarity on TLC to lucernic acid. Dicarboxylic sapogenins gave greenish colors whereas monocarboxylic aglycones appeared as purple spots.

Monosaccharide mixtures obtained from acid hydrolysis of saponin fractions were reduced, acetylated to the corresponding alditol acetates and identified by GLC using an authentic alditol acetate standard as reference. In hydrolysis experiments with known saponin and oligosaccharides the best hydrolysis conditions in terms of complete cleavage and minimal decomposition were determined. The fraction A (Table 3) on hydrolysis furnished only monosaccharides and no aglycone, hence it is not a saponin but may be an oligosaccharide. Uronic acids did not withstand the hydrolysis conditions unless lower acid concentrations similar to those used for graded hydrolysis experiments were applied. Five monosaccharides were encountered in saponin fractions of both alfalfa cultivars viz. glucose, galactose, xylose, arabinose and rhamnose. These sugars were also found in earlier studies.[8]

The distribution of the sugars showed remarkable differences between the saponins of Lahontan and DuPuits alfalfa (Table 3). It appears that there are no two identical fractions in the saponin mixtures of the two cultivars. The sugar values in Table 3 describe only the relative ratio of monosaccharides, not the actual number of molecules in a saponin, but remain significant, even though most of the saponin fractions are still mixtures.

TABLE 3. SAPOGENIN AND SUGAR COMPONENTS FROM THE HYDROLYSIS OF DUPUITS AND LAHONTAN ALFALFA SAPONIN FRACTIONS

Fraction no.	Sapogenin*	D-Glucose	D-Galactose	D-Xylose	L-Arabinose	L-Rhamnose†
DuPuits A	None	†	—	—	—	—
Lahontan A	None	†	†	—	—	—
DuPuits B1	**1**, **2**	2·40	—	0·92	1·05	1·00
Lahontan B1	**3**	3·02	—	0·96	1·14	1·00
DuPuits B1, B2	**1**, **2**	3·13	—	1·17	1·15	1·00
Lahontan B2	**2**	2·09	—	0·63	0·85	1·00
DuPuits C1, C2	**1**, **2**	2·36	—	0·86	1·18	1·00
Lahontan C	**3**	2·60	0·94	tr	1·19	2·00
DuPuits D	**1**, **2**	0·82	—	0·65	1·01	1·00
Lahontan D1	**3**, **4**, **5**	2·85	1·25	tr	1·04	3·00
Lahontan D2	**3**, **4**, **5**	1·42	0·39	0·31	0·48	2·00
DuPuits E1	**1**–**6**	2·60	tr	0·75	1·13	2·00
Lahontan E1	**3**, **4**, **6**	2·80	1·09	0·27	0·64	2·00
DuPuits E2	**3**, **4**, **6**	1·75	1·08	0·98	1·12	2·00
Lahontan E2	**6** (**3**, **4**)	1·86	0·27	tr	tr	1·00
DuPuits F1	**1**–**8**	1·00	—	tr	tr	tr
Lahontan F1	**4**, **6**, **3**, **7**, **8**	1·00	tr	tr	tr	tr
DuPuits F2	**1**	1·00	—	tr	tr	tr
Lahontan F2	Unknown	1·00	—	tr	tr	tr

* Sapogenins are listed in order of decreasing abundance. The designation is adopted from Table 2.
† Sugars are given in molar ratios, with L-rhamnose as standard.

Another striking difference between the cultivars is the prevalence of soyasapogenol A (**3**) in Lahontan saponins, where it was found in 6 fractions as compared to only 3 DuPuits fractions. These findings are even more significant because the major DuPuits aglycones (**1** and **2**) are so markedly different in structure from the prevalent Lahontan aglycone (**3**). While (**1**) and (**2**) incorporate 2 carboxyl substituents, **3** does not contain any acid groups. Apparently the two alfalfa cultivars have the capability of oxidizing C_{23} and C_{24} in a specific way. On the other hand it is apparent that sapogenins with one carboxyl group (**4** and **5**) are encountered predominantly in Lahontan saponins.

The distribution of the sapogenins in the different alfalfa cultivars suggest the concept of a cultivar-selective mechanism for the biosynthesis of particular triterpenoid sapogenins. The Lahontan and DuPuits alfalfa cultivars can be considered as two separate chemotypes [see results with *Withania somnifera* (L.) Dunal[12]] in that DuPuits, because of its enzyme system, is capable of synthesizing medicagenic acid, while the enzyme system in Lahontan is obviously modified and forms either the neutral soyasapogenols or monocarboxylic triterpenoids. That the actual process may be quite complex can be concluded from the observation that soyasapogenol A (**3**) seems to occur as a major aglycone only in fractions containing appreciable amounts of galactose, while sapogenins with two carboxyl groups (**1** and **2**) are found in saponin fractions that incorporate quantities of xylose.

[12] ABRAHAM, A., KIRSON, T., GLOTTER, E. and LAVIE, D. (1968) *Phytochemistry* 7, 957.

A more detailed investigation would require further resolution and purification of alfalfa saponins as suggested above. However, the present study already provides sufficient information for the evaluation of plant-breeding experiments[2] and of testing alfalfa saponin fractions against plant pathogens.[13] The significance of the numerous saponin compounds in relation to *in vivo* biological activity is unknown. *In vitro* studies have demonstrated that certain saponins are more toxic than others to test organisms in culture plates.[6]

Insect and disease resistance in DuPuits and Lahontan alfalfa is as contrasting as the saponin compounds.[9] Lahontan alfalfa is resistant to bacterial wilt [*Corynebacterium insidiosum* (McCull.) H. L. Jens], stem nematodes [*Ditylenchus dispachi* (Kühn) Filipjev], and the spotted alfalfa aphid, *Therioaphia maculata* (Buckton), and susceptible to foliage diseases, whereas DuPuits alfalfa is susceptible to bacterial wilt, stem nematodes, and the spotted alfalfa aphid, and fairly resistant to most foliage diseases. It should be emphasized that saponins or variations in saponins have not been linked with the insect- and disease-resistance patterns in the two cultivars. However, it is tempting to speculate that the contrasting chemotypes of the two cultures are somehow linked with resistance to some disease and insect pests.

In a general sense, the fact that DuPuits saponin has higher activity than that of Lahontan can probably be attributed to the fact that the aglycones in DuPuits are largely medicagenic acid, whereas those in Lahontan are largely soyasapogenol. This follows from the results of several workers, which suggest that saponin exerts its fungicidal effect by precipitating sterols or sterol compounds in the cell wall, resulting in ion leakage.[14–16]

Gestetner *et al.*[15] reported that lucerne saponins that contain medicagenic acids can be precipitated with sterols and have biological activity, whereas the soyasapogenol containing saponins, which are not precipitated with sterols, do not have biological activity. Tschesche and Wulff[17] were unable to associate the antimicrobial activity of saponins with acid or basic groups on the aglycone or the sugar groups, but they rated high activity for the alkaloid glycosides, tomatin, soladulcidintetraosid, and solanin (from *Solanum* species). Schlösser[16] concluded that the membranolytic action of saponins and the polyene antibiotics was associated with cell membrane sterols.

Gestetner *et al.*[18] found that an intact steroid ring structure having the conformation of cholestanol, to which a side chain characteristic of cholesterol or phytosterols is attached, is essential for the formation of a sterol-saponin addition product with lucerne saponin.

EXPERIMENTAL

For analytical TLC, 0·2 mm layers of silica gel G and a double development in BuOH–HOAc–H_2O (4:1:2) were used. Preparative TLC was performed on 1 mm silica gel HR layers using a single development in BuOH–HOAc–H_2O (4:1:2). Alditol acetates were analyzed by GLC on a 3% ECNSS-M column* (3·60 × 3 mm). High resolution MS of acetylated and methylated aglycones were recorded on an AEI-902 instrument.* The probe was 180–200°. Elemental formulae of parent ions were determined by peak matching.

* Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

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[16] SCHLOSSER, E. (1972) *Phytopath. Z.* **74,** 91.
[17] TSCHESCHE, R. and WULFF, G. (1965) *Z. Naturforsch.* **20,** 543.
[18] GESTETNER, B., ASSA, Y., HENIS, Y., TENCER, Y., ROTMAN, M., BIRK, Y. and BONDI, A. (1972) *Biochem. Biophys. Acta.* **270,** 181.

Preparation of saponin mixtures. Alfalfa (*Medicago sativa* L.) meal was produced on field plots at Logan, Utah. Forage was harvested prior to flowering, dried at 68° in a forced-air drier, ground to pass an 18-mesh screen in a hammer mill and then extracted in a continuous flow extractor with 95 l. of 95% EtOH for 5 days. The EtOH extract was fractionated as described by Wall *et al.*[10] The resulting BuOH extract was reduced in vol. by one-third and cooled to 20° and the precipitated saponin mixture removed by filtration and freeze-dried.

Preparative TLC separation. The crude saponin mixture (100 mg), dissolved in H_2O (0·4 ml), was applied evenly to a preparative TLC plate and developed. Saponin zones were visualized by the greased plate method,[19] eluted with MeOH–H_2O (2:1), and freeze-dried. After TLC analysis, each saponin fraction was subjected to further preparative TLC purification, using the acidic solvent system, until a chromatographically homogeneous product was obtained. The yields were 50–60% for each separation step.

Hydrolysis procedure. The purified saponin fraction (10 mg) was dissolved in 0·4 N H_2SO_4 (3 ml) and heated in an autoclave at 125° for 2·5 hr. This led to a ppt. S and an aq. soln M. S was removed by filtration, methylated (CH_2N_2), acetylated [C_5H_5N–$(Ac)_2O$, 1:1], and subjected to MS analysis. Authentic samples of medicagenic acid diacetate dimethylester, lucernic acid triacetate methylester, soyasapogenol B triacetate, and soyasapogenol A tetracetate served for comparison. Modifying a procedure described by Crowell *et al.*,[20] the soln M was neutralized with $BaCO_3$, reduced with $NaBH_4$ (20 mg), deionized with Dowex 50W-X4,* and evaporated to dryness. Two 5-ml portions of MeOH were evaporated from the residue, and the remainder was acetylated by heating with C_5H_5N–$(Ac)_2O$ (1:1) at 100° for 30 min. A $CHCl_3$ soln of the product was analyzed by GLC. An authentic mixture of 12 known alditol acetates served as standard. For graded hydrolysis, the saponin fraction was autoclaved with 0·2 N H_2SO_4 at 115° for 1 hr. The work-up of the hydrolysate was the same as that used before.

TLC resolution of aglycones and saponins. A sample (100 mg) of total saponin mixture was hydrolyzed as before. The resulting aglycone mixture was resolved on analytical TLC plates using $CHCl_3$–Me_2CO (9:1). The sapogenins were detected by spraying with a 5% soln of $CeSO_4$ in 20% H_2SO_4 and heating at 120°. The reference compounds used for MS were run as markers.

Saponin fractions which had been resolved by preparative TLC with BuOH–HOAc–H_2O were subjected to TLC analysis using $CHCl_3$–MeOH–H_2O (65:38:10). The developed plates were sprayed with $CeSO_4$–H_2SO_4 soln and carefully heated at 120° until the characteristic colors for each saponin appeared.

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