Alfalfa Saponins and Their Implication in Animal Nutrition

Sucharita Sen, Harinder P. S. Makkar, and Klaus Becker*

Institute for Animal Production in the Tropics and Subtropics (480), University of Hohenheim, D-70593 Stuttgart, Germany

Alfalfa (*Medicago sativa* L.) is of immense importance as livestock fodder for both developed and lesser developed countries as it contains a high amount of protein, and yield of dry matter is also very high. The main antinutritional components present in this plant are saponins, and their unfavorable effects on animal performance have restricted the optimum use of this high-protein plant as an animal feed. The occurrence, chemistry, analysis, and consequences of intake of alfalfa saponins are reviewed. The information synthesized may lead to planning of more detailed studies on isolation and characterization of saponins and sapogenins to gain a better understanding of the biological activities of the aglycon and carbohydrate moieties of saponins.

Keywords: *Alfalfa; Medicago sativa; saponins; animal nutrition; ruminants; nonruminants*

INTRODUCTION

The efficiency of animal production is dependent upon the optimum utilization of the feed for growth, development, and reproduction. A wide variety of feeds and feedstuffs of different origin are used in animal production. The nutritional value of feed is influenced by a number of factors, and these can affect the efficiency with which the feed is utilized to meet the animal's particular requirement. However, the presence of various antinutritional factors in livestock feed lowers the nutrient utilization, food conversion efficiency, and animal productivity. These antiquality factors can be divided into four groups: (1) factors affecting protein utilization and depressing digestion (protease inhibitors, tannins, saponins, lectins, etc.); (2) metal ion scavengers (oxalate, phytate, gossypol pigments, glucosinolates, etc.); (3) antivitamins (anti-vitamin A, anti-vitamin E, anti-vitamin D, dicoumarol, etc.); (4) factors other than those in the preceding categories (mycotoxins, mimosine, cyanogens, nitrates, alkaloids, photosensitizing agents, isoflavones, etc.) (Makkar, 1993). Much emphasis is given to various antinutritional factors that are most widespread in nonconventional feedstuffs, which are the tannins and saponins. Plants use these chemicals to defend themselves against environmental vagaries. The effective utilization of nonconventional foods should be the major area of research in the lesser developed countries due to shortage of conventional feedstuffs. Alfalfa is of immense importance for both developed and lesser developed countries as it contains a high amount of protein, and the yield of protein per unit area is greater than for any other known conventional crop. Alfalfa is used as a forage or industrially used to yield leaf protein concentrate, which is well balanced in amino acids and is rich in vitamins, carotenoids, and xanthophylls (Gastineau et al., 1981). Increased interest is now developing for more effective utilization of alfalfa as animal feed. The main antinutritional factor in this plant is saponin. Unfavorable effects of alfalfa saponins on animal performance have restricted the optimum use of this high-protein plant as an animal feed. A great deal of attention has been paid to saponins in alfalfa, primarily because of their antinutritional and physiological properties. Saponins have always been considered as deleterious by animal scientists. However, lately, a number of studies have shown both beneficial and adverse effects of saponins (Price et al., 1987; Cheeke, 1996). Our studies on beneficial effects of quillaja and yucca saponins on ruminants (Makkar et al., 1995; Makkar and Becker, 1996, 1997; Bosler et al., 1997) generated interest in the exploitation of beneficial effects, if any, of alfalfa saponins. The aim of the present paper is to encompass aspects of the occurrence, chemistry, analysis, and consequences of intake of this plant saponin and their physiological effects. It is hoped that the information may lead to planning of studies on the isolation of saponins for applications similar to yucca, quillaja, or ginseng saponins or on the evaluation of diets containing alfalfa or alfalfa saponins at low levels for livestock. Some methods commonly used for the quantification of saponins in this plant are also presented.

CHEMICAL ASPECTS OF ALFALFA SAPONINS

Saponins, the secondary metabolites of plants, are the naturally occurring sugar conjugates of triterpenoids or steroids possessing the properties of forming stable froth when shaken with water.

The most commonly occurring sapogenins of the alfalfa plant are of oleanane skeleton. Various attempts have been made to isolate and characterize saponins and sapogenins from this plant. Djerassi et al. (1957) showed that the sapogenol present in the dehydrated alfalfa meal is medicagenic acid (olean-12-ene-2β,3β-diol-23,28-dioic acid). Another compound, lucernic acid, was also isolated from this plant (Livingstone, 1959). Hydrolysis of the sterol-precipitated saponin fraction revealed the presence of hederagenin (olean-12-ene-3β,-23-diol-28-oic acid) (Shany et al., 1970a, 1972) along with medicagenic acid. West (1979) reported the presence of oleanolic acid (olean-12-en-3β-ol-28-oic acid) from the hydrolysate of sterol-precipitated saponins from alfalfa roots. In addition, alfalfa tops and roots have been shown to contain, after hydrolysis, a range of

S0021-8561(97)00389-0 CCC: $15.00
Published on Web 01/19/1998

soyasapogenols as sapogenins. Potter and Kummerrow (1954) identified soyasapogenols A, B, and C, while Shany et al. (1970b) reported soyasapogenols D and E. Jurzysta (1982) and later Oleszek and Jurzysta (1986) have reported the presence of soyasapogenols A−F, medicagenic acid, and hederagenin in the hydrolyzed extract of *Medicago media*.

The structures of the hydrolysis products of alfalfa saponins have been reinvestigated by Massiot et al. (1988a). They identified the soyasapogenols A−C and E, hederagenin, and medicagenic acid as known products. Bayogenin and zanhic acid, previously known from other sources, have also been characterized in alfalfa by them. The elusive lucernic acid was presumed to be a lactone artifact derived from zanhic acid (16-hydroxymedicagenic acid). Their structures have been established using modern spectroscopic techniques such as IR, NMR, and MS measurements (Massiot et al., 1988a).

Until the 1970s, the only saponin from alfalfa tops and root that had been fully characterized was 3-*O*-[*β*-D-glucopyranosyl(1→6)-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl]olean-12-ene-2*β*,3*β*-diol-23,28-dioic acid (Gestetner, 1971).

Morris et al. (1961) had isolated 3-*O*-[*β*-D-glucopyranosyl]olean-12-ene-2*β*,3*β*-diol-23,28-dioic acid from a partially hydrolyzed extract of alfalfa roots. A more complex saponin was later isolated from alfalfa flowers and shown to be 3-*O*-[*β*-rhamnopyranosyl-*β*-glucuronopyranosyl-*β*-glucopyranosyl]olean-12-ene-2*β*,3*β*-diol-23,-28-dioic acid; the linkages of the saccharide chain were not determined. The saponin composition of alfalfa is very complex. Using flash chromatography, Timbekova and Abubakirov (1984) have detected 13 saponins possessing the petacyclic triterpene aglycon, and Nonaka has separated 8 saponin components (Nonaka, 1986), 7 of which appeared to contain medicagenic acid. Timbekova and Abubakirov (1984) have also isolated and identified 3-*O*-[*β*-D-glucopyranosyl]olean-12-ene-2*β*,3*β*-diol-23,28-dioic acid (medicoside A) and its 28*β*-D-glucopyranol ester (medicoside G) from alfalfa roots of Central Asia. Medicoside C has been identified as 3-*O*-[α-L-arabinopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl]olean-12-ene-3*β*,23*β*-dioloic acid (Timbekova et al., 1985).

The medicagenic acid glycosides (at least 11 of which were separable by two-dimensional TLC) containing glucose, arabinose, xylose, and rhamnose comprised 6% of the dry weight of the root and showed wide-ranging biological activity, e.g., lysis of red blood cells, inhibition of *Trichoderma viride* growth, and retardation of the development of wheat seedlings (Price et al., 1987). In contrast to its leaves, roots, and flowers, alfalfa seeds have not been found to possess medicagenic acid-containing saponins; soyasapogenols B, C, and E (but not A and D) have been identified in the hydrolysate of the seed extract (Table 2). In addition, two unidentified aglycons were observed together with galactose and glucuronic acid (in largest amounts), glucose, rhamnose, and traces of xylose and arabinose (Jurzysta, 1973).

Massiot et al. (1991) have isolated three saponins from the leaves of alfalfa (cultivar Resis), and their structures were established as 28-*O*-[*β*-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl(1→2)-α-arabinopyranosyl]-medicagenate, 28-*O*-[*β*-D-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl]-3-*O*-[*β*-D-glucopyranosyl]medicagenate, and 28-*O*-[*β*-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl]-3-*O*-[*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl]-medicagenate; two saponins were isolated from the North American cultivar Lohantan, and their structures were determined as 28-*O*-[*β*-D-xylopyranosyl)-(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl], 3-*O*-[*β*-D-glucuronopyranosyl]medicagenate, and 3-*O*-[α-L-rhamnopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)-*β*-D-glucuronopyranosyl]soyasapogenol B. Structure elucidations were performed on peracetylated derivatives of saponins by using ^{1}H and ^{13}C NMR with techniques such as COSY, relayed COSY, HOHAHA, ROESY, HMBC, and HMQC.

From the reports so far obtained, it is evident that medicagenic acid glycosides could be found in both monodesmosidic and bisdesmosidic forms, with the latter being more abundant. Moreover, it can be recognized that the medicagenic acid glycosides can be divided into two distinct groups that are substituted at the 3-O position of the sapogenin with either glucose or glucuronic acid. The glucuronic acid-substituted medicagenic acid glycosides have been previously reported in alfalfa roots only in 1990 (Oleszek et al., 1990a).

The saponins isolated so far from varieties of alfalfa are represented in Table 1.

OCCURRENCE AND DISTRIBUTION

Reports on varietal differences in the saponin content of alfalfa stimulated interest in saponins as biologically active compounds. Saponin content of alfalfa was affected by variety, stage of growth, and number of leaves (Hanson et al., 1963). Seeds and leaves of *M. lupulina* were higher in saponin content than stems and flowers (Gorski et al., 1984). Immature plants were found to have more saponin content than comparitively more mature plants (Alder et al., 1985; Livingstone et al., 1979; Fenwick et al., 1983).

Root saponins were found to be more hemolytic than foliage saponins and also more inhibitory to the growth of *Tribolium castaneum* lervae. Saponins are found to be more concentrated in the outermost layer of the cortex of the root and decreased inwardly. Toxicity of the extracted saponins to insects and pathogens, higher content of saponins in the immature plants than in the mature (immature plants being more susceptible to environmental attacks), and storage of saponins in growing parts suggest that saponins are involved in resistance mechanisms (Birk, 1969). Alfalfa seed saponins lack medicagenic acid, and as a result they do not hemolyze red blood cells (Jurzysta, 1973).

Hanson et al. (1963) found differences in the saponin contents in the alfalfa cultivars Buffalo, Lahontan, Ranger, and Vernal from eight locations in the United States, and Pedersen et al. (1966) reported variations in the biological activity of saponins from alfalfa cultivars DuPuits, Lahontan, Ranger, and Uinta. The DuPuits cultivar was found to be higher in saponin content than cv. Lohantan, and this difference became greater as the growing season progressed (Pedersen et al., 1967).

Notable differences between the two cultivars are the respective contents of saponins containing soyasapogenols and medicagenic acid. In Lahontan the former predominated (45% vs 5%) and the reverse occurred in DuPuits (20% vs 40%). The lower levels of medicagenic acid-containing saponin in Lahontan are consistent with its improved nutritional value (Price et al., 1987). Six

Table 1. Saponins from Alfalfa

genin part	sugar constituent	available in plant part	common name	reference
medicagenic acid	3-*O*-Glu	roots		Morris et al. (1961)
	3-*O*-Glu, 28-*O*-Glu	roots	medicoside G	Oleszek et al. (1990a)
	3-*O*-[Glu(1→4)-Glu]	roots		Levy et al. (1989)
	3-*O*-[Glu(1→6)-Glu(1→3)-Glu]	roots		Gestetner (1971)
	3-*O*-[Rha-GluA-Glu]	flowers		Morris and Hussey (1965)
	3-*O*-Glu, 28-*O*-[Xyl(1→4)-Rha(1→2)-Ara]	roots and tops	medicoside J	Oleszek et al. (1990a)
	3-*O*-[Glu(1→2)-Glu], 28-*O*-[Xyl(1→4)-Rha(1→2)-Ara]	roots and tops		Oleszek et al. (1990a)
	3-*O*-Glu, 28-*O*-[Rha(1→2)-Ara]	roots	medicoside H	Timbekova et al. (1989)
	3-*O*-[Glu(1→2)-Glu{(1→3)Rha}(1→2) Glu], 28-*O*-Glu	roots	medicoside L	Timbekova et al. (1990)
	3-*O*-Glu A	roots		Oleszek et al. (1990a)
	3-*O*-Glu A, 28-*O*-[Xyl(1→4)-Rha(1→2)-Ara]	roots and tops		Oleszek et al. (1990a)
	3-*O*-[Gal(1→2)-Glu], 28-*O*-Glu	roots		Massiot et al. (1988b)
	3-*O*-[Rha(1→2)-Glu(1→2)-Glu], 28-*O*-Glu	roots		Massiot et al. (1988b)
	28-*O*-[Xyl(1→4)-Rha(1→2)-Ara]	tops		Massiot et al. (1991)
	3-*O*-Glu A, 28-*O*-[Rha(1→2)-Ara]	tops		Oleszek et al. (1992)
hederagenin	3-*O*-[Glu(1→2)-Ara], 28-*O*-Glu	roots	medicoside F	Timbekova et al. (1996)
	3-*O*-[Ara(1→2)-Glu(1→2)-Ara]	roots	medicoside C	Timbekova and Abubarikov (1985)
	3-*O*-[Glu(1→2)-Ara]	roots	caulosaponin B	Oleszek et al. (1990a)
	3-*O*-[Ara(1→2)-Glu(1→2)-Ara], 28-*O*-Glu		medicoside I	Massiot et al. (1988b)
	3-*O*-[Gal(1→2)-Ara]	roots		Massiot et al. (1988b)
	3-*O*-[Glu(1→3)-Xyl], 28-*O*-Glu	roots	medicoside E	Timbekova et al. (1996)
soyasapogenol B	3-*O*-[Rha(1→2)-Glu(1→2)-Glu]	leaves		Jurzysta (1973)
	3-*O*-[Glc(1→2)-GlcA]	tops		Kitagawa et al. (1988)
	3-*O*-[Rha(1→2)-Glc(1→2)-GlcA]	tops		Massiot et al. (1991)
	3-*O*-[Rha(1→2)-Gal(1→2)-GlcA]	tops and roots		Oleszek et al. (1990a)
soyasapogenol E	3-*O*-[Rha(1→2)-Gal(1→2)-GlcA]	tops		Kitagawa et al. (1988)
	3-*O*-[Rha(1→2)-Gal(1→2)-GlcA] (as maltol conjugate)	seeds		Massiot et al. (1992)
zanhic acid	3-*O*-Glc-Glc-Glc, 23-*O*-Ara, 28-*O*-Ara-Rha-Xyl-Api	tops		Oleszek et al. (1992)
	3-*O*-Glc-Glc-Glc, 23-*O*-Ara, 28-*O*-Ara-Rha-Xyl	tops		Oleszek et al. (1992)

Table 2. Genin of Alfalfa Saponin and Its Occurrence in Plant Parts

genin	availability in plant parts
soyasapogenol C	roots, seeds
soyasapogenol E	roots
soyasapogenol B	leaves, seeds
hederagenin	roots, leaves
bayogenin	roots, leaves
medicagenic acid	roots, leaves
lucernic acid	roots, leaves
zanhic acid	leaves
oleanolic acid	roots
soyasapogenol A	roots, tops
soyasapogenol D	roots, tops
soyasapogenol F	roots, tops

Lahontan fractions of saponins incorporated varying quantitites of galactose, which was present in only two DuPuits fractions. More arabinose was found in DuPuits than in Lahontan saponins. Presence or absence of medicagenic acid seemed to explain most of the differences in the biological activities of DuPuits and Lahontan. However, medicagenic acid is not the only sapogenin strongly active toward pathogens. An unidentified sapogenin chemically related to medicagenic acid inhibited fungal growth markedly but did not affect insects (potato leafhopper and pea aphid) (Horber et al., 1974). Differences in the nutritional and biological properties of high- and low-saponin cultivars of alfalfa prompted Berrang et al. (1974) to examine their respective saponin content. Chemical fractionation and TLC procedures revealed 33 saponins in DuPuits and 27 in Lahontan. Hydrolysis and investigation of sapogenol fractions from both of these cultivars by mass spectrometry revealed eight aglycons including soyasapogenols A and B, medicagenic acid, and lucernic acid. In retrospect, an additional aglycon may be seen to be hederagenin. Certain fractions containing medicagenic acid were responsible for toxic properties in all assays used (Horber et al., 1974). Tava et al. (1993) ascribed the contrasting response of DuPuits and Equipe cultivars to the hemolytic test to the significantly higher medicagenic acid found in the aerial parts of the DuPuits cultivar. Presence or absence of medicagenic acid seemed to explain most of the differences in biological activity of DuPuits and Lahontan or Equipe cultivars.

METHODS FOR QUANTIFICATION OF SAPONINS IN FEEDSTUFF

There is urgent need to develop simple and specific methods for quantification of saponins and to improve the existing one. Some methods that are in use for quantification are hemolysis, piscicidal activity, *T. viride* assay, gravimetry, spectrophotometry, TLC, GLC, and HPLC.

Hemolysis Methods. Hemolysis, i.e., the ability of saponins to rupture erythrocytes, has been used for decades as a detection and quantification method. Various quantitative methods using hemolysis have been reviewed by Birk (1969).

The European Pharmacopeia uses as a unit the quantity in milliliters of ox blood that is totally hemolyzed by 1 g of test substance. As a standard the saponin mixture from the root of *Gypsophila paniculata* (Caryophyllaceae) has by definition an activity of 30 000. The hemolytic index (HI) is calculated as

$$HI = 30000a/b$$

where *a* is the quantity of standard saponin in grams required for complete hydrolysis and *b* is the quantity of substance in grams required for complete hydrolysis. However, the hemolytic methods have the disadvantage that they rely only on the complete absence of other surface active compounds in the plant, which may also be hemolytic, and no simple chemical test exists for differentiating among the saponins with respect to their hemolytic activity (Hostettmann et al., 1995).

It was evident from the study of Oleszek (1990) that the hemolytic activities of the individual saponins are strongly structure dependent. Also, the technique used for the hemolytic test was shown to be important as the same compound showed different activities depending on the technique used.

The colorimetric method for determination of hemolysis, in principle, is based on the hemolytic index test procedure (Plhak, 1983). In another method, which is called the hemolytic micromethod, blood is mixed with gelatin and spread over glass plates as a uniform 0.5 mm thin layer. The width of the hemolytic ring observed after 20 h of spotting a saponin solution or alfalfa juice on the glass plate is a measure of saponins (Jurzysta, 1979). This method is simple, quick, and ideal for use in breeding programs (Oleszek, 1990).

It was shown that individual saponins gave different hemolytic activities depending on their structure and also on the hemolytic test used. Generally, it was found that saponin monodesmosides were more active than their bisdesmosidic analogues. These differences in hemolytic activities may strongly influence the results of saponin determination with hemolysis-based methods. It appears that though this method has been in use for a long time, there is significant controversy associated with it. This method can be used as a preliminary tool for the quantification of saponin mixture from any particular plant in which the standard should be any one of the purified saponins existing in that mixture.

Quantification by HPLC and GC. HPLC, because of its speed, sensitivity, and adaptability to nonvolatile polar compounds, is ideal for the analysis of saponins and sapogenins. This procedure has great possibilities for a precise look into saponin composition and concentration in a plant material. The advantage of quantitative HPLC over photometric methods is that the amounts of the individual saponins in a mixture or extract can be determined. Adulterations are easier to discern. However, peak resolution of saponin mixtures on some reversed phase HPLC columns sometimes is insufficient, and hydroxyapatite columns, chemically modified porous glass columns, silica gel columns, and HPLC of borate complex (specially used for mono- and oligosaccharides) are required for better resolution (Hostettmann et al., 1995).

The use of this new, more dependable analytical technique for saponin determination is limited because of difficulties with detection of triterpene saponins, which do not contain a UV chromophore and due to the lack of an appropriate standard. There have been several attempts to overcome the detection problems, which include detection of underivatized saponins at 190−200 nm (Domon et al., 1984) and monitoring with a light scattering detector (Ireland and Dziedzic, 1986). These modes of detection, however, have some limitation as to the solvents and gradients that can be used. Mono- and bisdesmosidic olean-12-ene saponins from alfalfa roots can be easily derivatized with 14-bromophenacyl bromide to produce UV-absorbing compounds, and the derivatized saponins were chromatographed in a single run on silica C_{18} (Oleszek et al., 1990b). Use of a refractive index detector in HPLC may also solve the problem. It was also shown by Ireland and Dziedzic (1986) that the use of HPLC with the mass detector (an evaporative light scaterring detector) can provide insight into the presence and extent of saponins in a saponin mixture.

Identification and quantification of all the sapogenins has been carried out by Tava and Odoardi (1996) using GC and GC/MS analysis. Sapogenins released after hydrolysis were methylated and acetylated to give corresponding products that are smoothly eluted under standard GC condition. This may be applied both to the quantification of individual glycosides and also for quantitative screening to find species with the highest content of the most biologically active compound for pharmaceutical purposes, such as fungitoxic activity against the medically important yeast *Candida, Torulopsis,* etc. This technique may also be used for the study of alfalfa top saponins for plant breeding and nutritional purposes as soon as appropiate standards are available.

Quantification by Color Reaction. A colorimetric method has been developed for the estimation of the ginseng saponin by Hiai et al. (1975). In this method it was reported that the saponin preparation and panaxadiol, an artifact sapogenin produced by acid hydrolysis (Shibata et al., 1962), gave a red-purple color when reacted with vanillin and sulfuric acid. It was suggested that the color reaction was applicable to a colorimetric determination of panaxadiol, estimated as saponin molar equivalent, in ginseng extracts. They proposed that the estimation using this procedure will become complicated and difficult with a saponin mixture containing oleanolic acid−saponin but the vanillin−sulfuric acid reaction may be applicable to alfalfa sapogenins and saponins, since the essential structure for the reaction is the −OH group at C-3 in free or glycosidic form (Hiai et al., 1976). The colorimetric method is easy, sensitive, and useful for the quantification of saponins, but it lacks specificity. This method for the estimation of saponins needs to be standardized using a purified saponin from alfalfa. Interfering moieties such as pigments might have to be removed from the alfalfa before preparation of the extract for saponin quantificaton.

Quantification by Thin Layer Chromatography (TLC). Quantitative TLC was used by Fenwick and Oakenfull (1983) to estimate the saponin content of 20 common food plants and also of foods prepared from some of them. A series of accurately known volumes of methanolic extract and also a standard solution of a purified saponin in the same solvent are spotted onto glass-backed plates, and the plates are developed with suitable solvent systems and sprayed with suitable spraying reagent. Saponins are quantified from intensities of the spots measured using a densitometer. This method can possibly be applied for the quantification of saponins present in major amounts in different varieties of the alfalfa using a purified saponin from alfalfa. Major biologically active saponins could also be identified by spraying the TLC plate with a suspension of red blood cells.

Saponin Bioassay. The bioassays of saponin are

mainly based on their activity against fungi. Of all the fungi tested, *T. viride* showed the highest sensitivity to the presence of saponins in the growth medium. The inhibition of *T. viride* is therefore used as a measure of saponin concentration of some plant extracts. Scardavi and Elliot (1967) claimed that the sugar chain of the saponin moiety is an important factor in determining its water solubility, i.e., an essential condition for activity against *T. viride*. Various trials with derivatized medicagenic acid and its glycosides proved that the activity of the saponins is markedly dependent upon free −COOH and −OH groups. It was also concluded that a free hydroxyl functionality at position C-3 is essential for antimycotic activity against *Sclerotium rolfsii*, *Fusarium oxysporum* f sp. *lycopersici*, *Rhizoctonia solani*, and *Aspergillus niger*. In addition, the nature of the aglycon portion of saponins is also important for the *T. viride* activity (Oleszek, 1996).

In the aerial parts of alfalfa the total amount of saponins determined by the *T. viride* test was 0.95% of dry matter, and this value strictly corresponds to the content of 0.94% dry matter of the biologically active fraction; however, this value excludes zanhic acid tridesmoside and soyasaponin I, which show no activity against *T. viride* (Oleszek et al., 1990a, 1992). This value correlated with the data reported by Jurzysta (1982) obtained for a number of alfalfa varieties. In this biologically active fraction, 3-glcA, 28-ara-rha-xyl predominated (60%), and this finding 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 saponin in addition to zanhic acid tridesmoside and soyasaponin I, which cannot be determined by *T. viride* assay. It is recognized, however, that other compounds present in the plant extract may also inhibit *T. viride* to a certain extent (Pedersen, 1975). This method also cannot therefore reliably be used for the quantitative determination of saponins in the animal feeds.

EFFECTS OF ALFALFA SAPONINS ON ANIMALS

Saponins may have significant effects on all phases of animal metabolism from ingestion of feed to the excretion of wastes. Ingested saponins have been observed to influence animal performance and metabolism in a number of ways. The biological activity of saponins depends not only on the structure of the lipophilic aglycon but also on the sugar composition. The three-dimensional spatial orientations of the saponins also play an important role in its bioactivity. The influence of legume saponins on animals has been widely reviewed by Cheeke (1983).

Effects on Ruminants. Alfalfa saponins have influence on rumen fermentation. Lu and Jorgensen (1987) showed that alfalfa saponins, isolated by ethanol extraction, hydrolyzed partially, and administered to sheep intraruminally, reduced microbial fermentation and nutrient digestion in the rumen. Total protozoal count was also significantly reduced. Apparent digestion coefficient of organic matter, hemicellulose, and cellulose in the total digestive tract were increased by saponins in sheep fed concentrate diets. Fractional digestion coefficients of organic matter, hemicellulose, cellulose, and nitrogen were reduced in the stomach, while they were increased in the small intestine by saponins in both concentrate diets and high roughage diets. Saponins also reduced total short-chain fatty acids and microbial protein synthesis in continuous flow cultures of rumen bacteria (Lu and Jorgersen, 1987).

The study of Lu et al. (1987) suggested that saponins may adversely affect microbial protein synthesis in the rumen. Lack of proportionality between concentration of saponin extract and microbial effects suggests that the relation between alfalfa saponins and microbial metabolism is not of the first order; factors other than saponin in the alfalfa extract are confounding the interpretation, as was shown in the in vivo experiment with the alfalfa saponin fed to sheep. Of particular interest was a marked reduction in rumen protozoa numbers, particularly with high concentrate diets.

Bloat in sheep was experimentally demonstrated to be the effect of intraruminal administration of alfalfa saponins. Production of slime from alfalfa saponins by rumen bacteria and alteration of the surface tension by rumen content were suggested as factors contributing to bloat formation. The development of high-saponin (HS) and low-saponin (LS) near-isogenic strains of alfalfa (Pedersen et al., 1973) has provided the opportunity to obtain new evidence on the saponin theory of bloat. Alfalfa saponins and their interaction with alfalfa proteins proved that alfalfa saponins do not contribute to pasture bloat by either the toxic or the foaming modes of action (Majak et al., 1980). The attention of most bloat research has been focused on the plant proteins as the major foaming agent responsible for bloat on legume pasture. However, a possible secondary role for saponins as bloat-causing agents has not been ruled out, and Cheeke recommended a full assessment of the interaction between saponins and other foaming agents in forages to clarify the possible role of saponins in bloat (Cheeke, 1976). Experimental bloat was produced in sheep by oral dosing with alfalfa saponins (Cowin et al., 1955). Lindahl et al. (1957) concluded that alfalfa saponins could cause ruminant bloat by toxic inhibition of reticulorumen activity as well as by foaming. Alfalfa saponins appear to be responsible for bloat under some circumstances. The circumstances/factors responsible for production of bloat by alfalfa saponins are not clear. Some proposals have been put forward to reduce bloat of cattle while using alfalfa as their feed (Majak et al., 1995; Majak and Hall, 1990, 1993; Howarth et al., 1977). Research is warranted in this area.

In the course of studies on the interactions of rumen microbes and alfalfa saponins, rumen microbes are likely to break the glycosidic bonds to release sugars and the aglycon, which might be considered as degradation of saponins. The aglycon molecule, being complex, will be difficult to break by rumen microbes. There is no evidence of complete breakdown of the aglycon part by the rumen microbes. These aglycons could have different effects and probably will not reflect the activity of the parent saponins. In reality, the prominent effects of saponins in ruminants may not be the effects of saponins themselves, but the effects of the degraded products of the saponins. *Butyrivibrio* strains were suggested to be able to degrade alfalfa saponins, but such degradation was not observed when the isolated strains (by nonenrichment technique) were used for such studies (Gutierrez et al., 1962). Chains of cocci were found to be the predominant type of bacteria in

several samples obtained from fermenters receiving saponins among the many morphotypes of bacteria present in the rumen. Gutierrez et al. (1958) suggested that the predominant type of bacterial species present in a saponin-containing medium was small, curved, Motile, rod-shaped, and Gram-negative.

Although alfalfa saponins have been assumed to be degradable by rumen microorganism, the ability of rumen bacteria to degrade feed to produce volatile fatty acid was apparently impaired in the presence of this alfalfa saponin-containing fraction. This may have been due to the disruption of cell membranes that results from precipitation of medicagenic acid with sterols in the cell membrane (Glauert et al., 1962). The molar proportion of propionate was increased by 1% saponins as compared with control treatment, and acetate to propionate ratio decreased in all saponin treatment in vitro studies of Lu et al. (1987).

Effects on Nonruminants. Retardation of growth by alfalfa dietary saponins has been observed in livestock and laboratory animals, probably due to the bitter and astringent sensory characteristics in the processed grain products. One mechanism that might account for the growth-depressing effects of saponins is the lowering of feed intake because of unpalatability (bitterness of saponins). Recent sensory test trials performed with human volunteers, using saponins isolated from alfalfa aerial parts, showed that zanhic acid tridesmoside is the most bitter astringent and throat-irritating compound of all coumpounds tested (Oleszek et al., 1992). This suggests that zanhic acid tridesmoside may be a major principle for bitterness of alfalfa and may influence palatability of the feed. Once swallowed, this saponin may irritate the membranes of the mouth and digestive tract. This may primarily influence the absorption of nutrients, but perhaps also allergens, xenobiotics, and other toxic dietary components might play an important role. In this respect zanhic acid seems to be a very important compound, too (Gee et al., 1997).

It was found that rats preferred diets with low saponin alfalfa at all levels of alfalfa tested (10, 15, 20, 25, and 30%) (Cheeke et al., 1977). In studies with geese, turkeys, quail, and chickens (Leghorn roosters) fed with HS and LS alfalfa meal, at levels 1−20% of the diet Cheeke et al. (1981) found that the only discrimination between the two alfalfa types was with geese fed 20% alfalfa. At this level they preferred the LS type. Rabbits showed no discrimination between the two types of alfalfa levels up to 30% of their diet. At higher levels they preferred the LS alfalfa (LeaMaster and Cheeke, 1979).

In a series of experiments, the effects of a range of structurally divergent alfalfa saponins on the potential difference across the rat small intestine were examined in vitro (Oleszek et al., 1994). Typically, there was an immediate reduction in potential diffference, although there was also considerable variation in response to particular compounds. Among the glycosides of medicagenic acid, bisdesmosides containing four sugar moities had activities equal to a monodesmoside and to medicagenic acid itself. The exception was 3,28-diglucoside medicagenic acid, which did not affect membrane potential. In another study by Oleszek (1996) zanhic acid tridesmoside was administered to hamsters via a stomach tube. The hamsters were observed to suffer from breathing problems during the first few hours of administration. After 10−15 h, animals suffered nervous system perturbations followed by death after 24 h. From the calculation of the LD_{50} value, zanhic acid was classified as a toxic/moderately toxic compound. It was also found that the intestines of the hamsters were heavily filled with gas after administration. As the hamsters were not fed for several hours before administration of the zanhic acid, the role of zanhic acid tridesmoside in bloat formation cannot be neglected. Zanhic acid tridesmoside again showed the highest activity of all saponins tested. The high depolarizing activity of zanhic acid glycosides suggested that the role of alfalfa saponins as antinutritional factors needs to be revised (Oleszek, 1996).

A reduction in blood plasma cholesterol occurred when saponins were fed to chicks and adult roosters (Griminger and Fisher, 1958), and a reduction in adrenal ascorbic acid resulted from a subcutaneous injection of saponin in rats (Vacek and Sedlak, 1962). The information on chicks and rats indicates that saponins inhibit growth rate, and investigations on the effects of feeding rations relatively high in saponin may be worthwhile. The significance of these results should be determined in relation to feed utilization. Peterson (1950) showed that addition of cholesterol or cottonseed oil to a diet containing 20% alfalfa meal prevented the growth depression otherwise produced in chicks on such a diet. Cookson and Federoff (1968) reported that hypercholesterolemia in rabbits induced by cholesterol feeding could be prevented by inclusion of alfalfa meal in diet. Malinow et al. (1978) found that feeding 50% alfalfa diets to cynomolgus monkeys following a period of cholesterol loading caused a decrease in cholesterolemia and plasma phospolipid levels, normalization in the distribution of plasma lipoproteins and reduction of aortic and coronary atherosclerosis. A significant reduction in the serum cholesterol level was observed when human volunteers consumed alfalfa seeds in a fruit juice suspension at meal time for three weeks (M. R. Malinow, P. McLaughlin, and C. Stafford, Oregon Regional Primate Center, Beaverton, OR, personal communication, 1980).

If it is assumed that the saponins in the alfalfa meal combine with fatty material in the intestines and are eliminated without being absorbed, alfalfa varieties high in saponin would be a disadvantage because of the elimination of fatty material with the saponin. Conversely, beneficial lowering of plasma cholesterol levels in humans has also been attributed to the saponins. The involvement with cholesterol leads to the possibility of an interference with other functions. Feeding alfalfa protein concentrate made from a HS strain of alfalfa is nutritionally undesirable for nonruminants. The mechanism by which alfalfa saponins interfere with cholesterol absorption has not been determined, although there is a longstanding view that they form insoluble complexes with cholesterol in the gut lumen (Coulson and Evans, 1960).

The toxicity of saponins to insects suggested that they protect the plant from insect predation. An appreciable quantity of partially purified saponin mixtures from alfalfa leaves was used by Tava and Odoardi (1996) to study the insecticidal activity. It was found that the increasing saponin rates added to the artificial diet of the European grape moth (*Lobesia botrana* Den & Schiff) caused an increased larval mortality. The saponin toxicity was thought to be exerted by the capacity of the saponins to form complexes with cholesterol,

which had been verified by the fact that the addition of cholesterol to the arificial diet reduced the mortality of the larvae. The lethal dose was found to be 1.342 ppm per larva. A similar effect was also found for other insects such as the summer fruit tortrix moth (*Adoxophyes orana* F.v.R), the European corn borer (*Ostrinia nubilalis* Hb.), *Tenebrio molitor*, and *Spodoptera littoralis*. These results may lead to the development of natural insecticide necessary for plant and crop protection. Crude alfalfa root saponins, their prosaponins produced by the alkaline hydrolysis of the total extract, and medicagenic acid sodium salt were tested in field trials against spider mite (*Tetranychus urticae* Koch.) and hop aphid (*Phoron humuli* Schrank). It was shown that the prosapogenins were most active against phytopathogen (Puszker et al., 1994).

Activity of the alfalfa saponins was also tested in human beings, and purified alfalfa saponins were found to inhibit the growth of human leukemic cell line K562 in vitro (Tava and Odoardi, 1996).

Interaction between the protein and saponin is poorly understood. It is of immense importance with respect to physical and physiological functionalities to characterize the interaction between proteins and saponins in alfalfa as both the protein and saponin contents are very high in this plant. From the studies of Ikedo et al. (1996) on the interaction of soyasaponin and bovine serum albumin, it has been concluded that the the N-terminal peptide fragment of bovine serum albumin interacts with soyasaponin to form a protease-resistant moiety with low sensitivity to α-chymotrypsin. The protease probe method on α-chymotrypsin, which examines the surface hydrophobicity of proteins, suggested that the aglycon moiety of saponin interacted with the surface hydrophobic region of bovine serum albumin. As there is much similarity in the chemical structure of the aglycon of the alfalfa saponins and soyasaponin, it may be assumed that the alfalfa saponins will also react with bovine serum albumin in the same way. During fractionation of the plant extract, it was also found that the concentration of saponins in the alfalfa protein concentrate was much higher. From this observation, it can be proposed that there is considerable interaction of the protein and saponin in the plant itself. The study of interaction of saponin and protein both isolated from alfalfa will be of much interest, which will lead to better insight into the proper utility of alfalfa and its fraction, for example leaf protein, as an animal feed.

Despite all of this work, little is known today about the structure−activity relationship of these saponins. As part of a co-operative program aiming at introducing new varieties of alfalfa and to establish the antinutritional/beneficial function of saponins, chemical structures of each and every component of the saponin mixtures from all parts of the plant are to be thoroughly investigated as all of these behavorial properties are related to certain saponin structures rather than to all members of the family.

CONCLUSION

Despite considerable amount of work done and firm views expressed by various workers in this field, it is not yet possible to reach a conclusion as to the usefulness and importance of saponins in animal nutrition. Much of the present uncertainty appears to be due to the fact that mixtures of saponins have often been treated as a single, well-defined substance. The concentrations of the individual saponins also were not known in a mixture, which is used very often for various studies. In the nutritional studies, the term saponin was often used only as an operational definition applied to mixtures of substances of unknown purity and chemical structures.

Genetic and environmental influences on saponins are still not known. Moreover, there is no information available on the effects of growth conditions or different growth phases on levels of individual saponins in the plant.

Modification of saponin content of alfalfa varieties might permit greater use of alfalfa as a forage than is at present. If heritability for saponin is high, the modification of saponin content by selection is an attractive possibility for the better utilization of alfalfa as an animal feed.

Extensive studies on the saponins are necessary to have a better insight into the nutritional aspects of alfalfa. Alfalfa saponins are composed of several bioactive fractions. Bioassay-guided fractionation of every part of the plant, isolation and characterization of the individual saponin components of each fraction, and study of effects of the purified saponins on animals may lead to proper utilization of alfalfa as animal feed or for other applications such as lowering of blood cholesterol levels.

If hemolytic methods are to be used for saponin determination, these need to be standardized with a saponin isolated from that particular plant material. The hemolytic activity of individual saponins is strongly structure dependent.

Methods based on HPLC, because of their sensitivity and adaptability to nonvolatile polar compounds, are ideal for analysis of saponins and sapogenins. However, for better peak resolution of saponin mixtures, proper choice of columns and solvent systems is required, which is determined by trial and error method.

Quantification of saponins by colorimetric method and by quantitative TLC method is expected to be the most useful method for routine quantification of saponins, as these are convenient and cheap and take less time. Using the small amount of pure saponin, total saponin content in a wide variety of species can be determined.

It seems that zanhic acid glycosides are important antinutritional factors in alfalfa 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 can be considered as an important glycoside as an antinutritional factor among alfalfa saponins. The HPLC procedure gives great possibilities for a precise look into saponin composition and concentration in the plant material (Nowacka and Oleszek, 1994). The presence or absence of medicagenic acid appears to explain most of the differences in the biological activity of the saponin fractions from different varieties of alfalfa, but it is also expected that zanhic acid tridesmoside has also a significant role in the determination of the biological activity of alfalfa saponins.

Alfalfa saponins possess the property of lowering the plasma cholesterol concentration by forming insoluble

complexes, and this may be considered as an important factor in human diets to reduce the risk of heart disease. As the cholesterol binding properties of saponins are strongly structure dependent, the actual mode of action of the individual saponins of alfalfa in cholesterol lowering is yet to be determined. An attempt has been made by Micich et al. (1992) to develop a polymer-supported saponin that can be used to remove cholesterol from high-cholesterol food and reused after the cholesterol has been washed out of the polymer support. Use of alfalfa saponins in this area is also a great prospect.

LITERATURE CITED

Alder, C.; Hiller, K. Bisdesmodische Triterpensaponine. *Pharmazie* **1985**, *40*, 676−679.

Berrang, B.; Davis, K. H., Jr.; Wall, M. E.; Hanson, C. H.; Pedersen, M. E. Saponin of two alfalfa cultivars. *Phytochemistry* **1974**, *13*, 2253−2260.

Birk, Y. Saponins. In *Toxic Constituents of Plant Food Stuffs*, 2nd ed.; Academic Press: New York, 1969; pp 161−182.

Bosler, D. A.; Blümmel, M.; Bullerdieck, P.; Makkar, H. P. S.; Becker, K. Einfluss eines saponinhaltigen Futterzusatzes auf Wachstumsverlauf und Schlachtkörperparameter heranwachsender Lämmer (Influence of a saponin containing feed addtive on mass development and carcass evaluation of growing lambs). *Proc. Soc. Nutr. Physiol.* **1997**, *6*, 46−47.

Cheeke, P. R. Nutritional and physiological properties of saponins. *Nutr. Rep. Int.* **1976**, *13*, 315−325.

Cheeke, P. R. Biological properties and nutritional significance of legume saponins. In *Leaf Protein Concentrate*; Telek, L., Graham, H., Eds.; AVI Publishing: Westport, CT, 1983.

Cheeke, P. R. Biological effects of feed and forage saponins and their impact on animal production. In *Saponins Used in Food and Agriculture*; Waller, G. R., Yamasaki, Y., Eds.; Plenum Press: New York, 1996; pp 377−386.

Cheeke, P. R.; Kinzell, J. H.; Pedersen, M. W. Influence of saponins on alfalfa utilization by rats, rabbits, and swine *J. Anim. Sci.* **1977**, *45*, 476−481.

Cheeke, P. R.; Powley, J. S.; Nakaue, H. S.; Arscott, G. H. Feed preferences of poultry fed alfalfa meal, high and low saponin alfalfa and quinine sulfate. *Proc. West. Sect. Am. Soc. Anim. Sci.* **1981**, *32*, 426−427.

Cookson, F. B.; Federoff, S. Quantitative relationships between administered cholesterol and alfalfa required to prevent hypercholesterolemia in rabbits. *Br. J. Exp. Path.* **1968**, *49*, 348−355.

Coulson, C. B.; Evans, R. A. The effect of saponin, sterol and linoleic acid on weight increase of growing rats. *Br. J. Nutr.* **1960**, *14*, 121−134.

Cowin, H. W.; Cupps, P. T.; Thompson, C. R. The effect of alfalfa saponin on rumen activity in sheep. *J. Dairy Sci.* **1955**, *38*, 606 (Abstr.).

Djerassi, C.; Thomas, D. B.; Livingstone, A. L.; Thompson, C. R. The structure and stereochemistry of medicagenic acid. *J. Am. Chem. Soc.* **1957**, *79*, 5292−5297.

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

Fenwick, D. F.; Oakenfull, D. G. Saponin content of food plants and some prepared foods. *J. Sci. Food Agric.* **1983**, *34*, 186−191.

Gastineau, I.; De Mathan, O. La Preparation Industriella de la Proteine Verte de Luzerne. In *Proteines Foliaires et Alimentation*; Costes, C., Ed.; Bordas: Paris, 1981; pp 159−182.

Gee, J. M.; Price, K. R.; Riout, C.; Johnson, I. T.; Fenwick, G. R. Effects of some purifeied saponins on transmural potential difference in mammalian small inte stine. *Toxicol. in Vitro* **1989**, *3*, 85−90.

Gee, J. M.; Wal, J. M.; Miller, K.; Atkinson, H.; Grigoriadou, F.; Wijnands, M. V. W.; Penninks, A. H.; Wortley, G. M.; Johnson, I. T. Effect of saponin on the transmucosal passage of β-lactoglobulin across the proximal intestine of normal and immunologically sensitized rats. *Toxicology* **1997**, *117*, 219−228

Gestetner, B. Structure of a saponin from lucerne (*Medicago sativa*). *Phytochemistry* **1971**, *10*, 2221−2223.

Glauert, A. M.; Dingle, J. T.; Lucy, J. A. Action of saponin on biological cell membranes. *Nature* **1962**, *196*, 952 −955.

Gorski, P. M.; Jurzysta, M.; Burda, S.; Oleszek, W. A.; Ploszyski, M. Studies on *Medicago lupulina* saponins. IV. Variation in the saponin content of *M. lupulina*. *Acta Soc. Bot. Pol.* **1984**, *53*, 543.

Griminger, P.; Fisher, H. Dietary saponins and plasma cholestrol in the chicken *Soc. Exp. Biol. Med. Proc.* **1958**, *99*, 424−426.

Gutierrez, J.; Davis, R. E. Isolation of saponin-digesting bacteria from the rumen of bloating cattle on ladino clover pasture. *J. Anim. Sci.* **1962**, *21*, 819−823.

Gutierrez, J.; Davis, R. E.; Lindahl, I. L. Dissimilation of alfalfa saponins by rumen bacteria. *Science* **1958**, *127*, 335.

Hanson, C. H.; Kohler, G. O.; Dudley, J. W.; Sorensen, E. L.;Van Atta, G. R.; Taylor, K. W.; Pedersen, M. W.; Carnahan, H. L.; Wilsie, C. P.; Kehr, W. R.; Lowe, C. C.; Stanford, E. H.; Yungen, J. A. Saponin content of alfalfa as related to location, cutting, variety and other variables *USDA Res. Rep. ARS* **1963**, 34−44.

Hiai, S.; Oura, H.; Odaka, Y.; Nakajima, T. A colorimetric estimation of ginseng saponins. *Planta Med.* **1975**, *28*, 363−369.

Hiai, S.; Oura, H.; Nakajima, T. Color reaction of some sapogenins and saponins with vanillin and sulfuric acid. *Planta Med.* **1976**, *29*, 116−122.

Horber, E.; Leath, K. T.; Berring, B.; Marcararian, V.; Hanson, C. H. Biological Activities of Saponin Components from DuPuits and Lahontan Alfalfa. *Entomol. Exp. Appl.* **1974**, *17*, 410−424

Hostettmann, K.; Marston, A. In *Chemsitry and Pharmacology of Natural Products: Saponins*; Phillipson, J. D., Ayres, D. C., Baxter, H., Eds.; Cambridge University Press: Cambridge, U.K., 1995; pp 125, 135.

Howarth, R. E.; Majak, W.; Waldern, D. E.; Brandt, S. A.; Fesser, A. C.; Goplen, B. P.; Spurr, D. T. Relationship between ruminant bloat and the chemical composition of alfalfa herbage. I. Nitrogen and protein fractions. *Can. J. Anim. Sci.* **1977**, *57*, 345−357.

Ikedo, S.; Shimoyamada, M.; Watanabe, K. Interaction between bovine serum albumin and saponin as studied by heat stability and protease digestion. *J. Agric. Food Chem.* **1996**, *44*, 792−795.

Ireland, P. A.; Dziedzic, S. Z. High performance liquid chromatography of soysaponins on silica phase with evaporative light scattering detection. *J. Chromatogr.* **1986**, *361*, 410−416.

Jurzysta, M. Isolation and chemical characterization of saponins from Leucerne seeds (*Medicago medis* Pers). *Acta Soc. Bot. Pol.* **1973**, *42*, 201−207.

Jurzysta, M. Haemolytic micromethod for rapid estimation of toxic alfalfa saponins. *Acta Agrobot.* **1979**, *32*, 5−11.

Jurzysta, M. Badania nad saponinami krajowych populacji lucerny mieszancowej (*Medicago medica* Pers.). *Wydawnictwa IUNG Ser. (Pulawy)* **1982**, *170*, 64.

Kitagawa, I.; Taniyama, T.; Murakami, T.; Yoshihara, M.; Yoshihara, M. Sapponin and sapogenol. XLVI. On the constituents in aerial part of American alfalfa, *Medicago sativa* L. The structure of dehydrosoysaponin I. *Yakugaku Zasshi* **1988**, *108*, 547.

LeaMaster, B. R.; Cheeke, P. R. Feed preferences of swine: Alfalfa meal, high and low saponin alfalfa and quinine sulfate. *Can. J. Anim. Sci.* **1979**, *59*, 467−469.

Levy, M.; Zehavi, U.; Naim, M.; Polacheck, I. Isolation, structure determination, synthesis and antifungal activity of a new native alfalfa root saponin. *Carbohydr. Res.* **1989**, *193*, 115−123.

Lindhal, I. L.; Davis, R. E.; Terttell, R. T.; Whilmore, G. E.; Shalkop, W. T.; Dougherty, R. W.; Thompson, C. R.; Van Atta, G. R.; Bickoff, E. M.; Walter, E. D.; Livingstone, A. G.; Guggolz, J.; Wilson, R. H.; Sideman, M. B.; DeEDS, F. Alfalfa saponins, studies on their chemical, pharmacological and physiological properties in relation to ruminant bloat. *USDA Tech. Bull.* **1957**, *1161*.

Livingstone, A. L. Lucernic acid, a new triterpene from alfalfa. *J. Org. Chem.* **1959**, *24*, 1567−1568.

Livingstone, A. L.; Knuckles, B. E.; Edwards, R. H.; deFremery, D.; Miller, R. E.; Kohler, G. O. Distribution of saponins in alfalfa protein recovery systems. *J. Agric. Food Chem.* **1979**, *27*, 362−366.

Lu, C. D.; Jorgensen, N. A. Alfalfa saponins affect site and extent of nutrient digestion in ruminants. *J. Nutr.* **1987**, *117*, 919−927.

Lu, C. D.; Tsai, L. S.; Schaefer, D. M.; Jorgensen, N. A. Alteration of Fermentation in Continuous Cultutre of Mixed Rumen Bacteria by Isolated Alfalfa Saponins. *J. Dairy Sci.* **1987**, *70*, 779−805.

Majak, W.; Hall, J. W. Sodium and patassium concentrations in ruminal contents after feeding bloat-inducing alfalfa to cattle. *Can. J. Anim. Sci.* **1990**, *70*, 235−241.

Majak, W.; Hall, J. W. Effect of time of feeding on the incidence of alfalfa bloat in cattle. *Proceedings, 22nd Conference on Rumen Function*, Chicago, IL; 1993, Abstract.

Majak, W.; Howart, R. E.; Fesser, A. C.; Goplen, B. P.; Pedersen, M. W. Relationships between ruminant bloat and the composition of alfalfa herbage II. Saponins. *Can. J. Anim. Sci.* **1980**, *60*, 699−708.

Majak, W.; Hall, J. W.; McCaughey, W. P. Pasture Management Strategies for reducing the risk of legume bloat in cattle. *J. Anim. Sci.* **1995**, *73*, 1493−1498.

Malinow, M. R.; McLaughlin, P.; Naits, H. K.; Lewis, L. A.; McNulty, W. P. Effects of alfalfa meal on shrinkage (regression) of atherosclerotic plaques during cholesterol feeding in monkeys. *Atherosclerosis* **1978**, *30*, 27−43.

Makkar, H. P. S. Antinutritional factors in foods for livestock. *Br. Soc. Anim. Prod.* **1993**, *16*, 69−85.

Makkar, H. P. S.; Becker, K. Degradation of Quillaja saponins by mixed rumen microbes. *Lett. Appl. Microbiol.* **1997**, *25*, 246−248.

Makkar, H. P. S.; Blümmel, M.; Becker, K. *In vitro* effects of and interactions between tannins and saponins and fate of tannins in the rumen. *J. Sci. Food Agric.* **1995**, *69*, 481−493.

Makkar, H. P. S.; Becker, K. Effects of Quillaja saponins on *in vitro* rumen fermentation. In *Saponins Used in Food and Agriculture*; Waller, G. R., Yamasaki, Y., Eds.; Plenum Press: New York, 1996; pp 387−394.

Massiot, G. C.; Lavaud, C. D.; Guillaume, D.; Men-Olivier, L. L. Reinvestigation of the Sapogenins and Prosapogenins from Alfalfa (*Medicago sativa*). *J. Agric. Food Chem.* **1988a**, *36*, 902−903.

Massiot, G.; Lavaud, C.; Men-Olivier, L. L.; Van Bist, G.; Miller, S. P. F.; Fales, H. M. Structural elucidation of alfalfa root saponins by mass spectrometry and nuclear magnetic resonance analysis. *J. Chem. Soc., Perkin Trans. 1* **1988b**, 3071−3079.

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

Massiot, G.; Lavaud, C.; Benkhaled, M.; Men-Olivier, L. L. Soyasaponin VI, a new maltol conjugate from alfalfa and soybean. *J. Nat. Prod.* **1992**, *55*, 1339−1342.

Micich, T. J.; Foglia, T. A.; Holsinger, V. H. Polymer supported saponins: An approach to cholesterol removal from butteroil. *J. Agric. Food Chem.* **1992**, *40*, 1321−1325.

Morris, R. J.; Hussey, E. W. A natural glycoside of medicagenic acid. An alfalfa blossom saponin. *J. Org. Chem.* **1965**, *30*, 166−168.

Morris, R. J.; Dye, W. B.; Gisler, P. S. Isolation, purification and structural identity of an alfalfa root saponin. *J. Org. Chem.* **1961**, *26*, 1241−1243.

Nonaka, M. Variable sensitivity of Trichoderma viride to Medicago sativa saponins. *Phytochemistry* **1986**, *25*, 73−75.

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

Oleszek, W. Structure Specificity of Alfalfa (*Medicago sativa*) Saponin Haemolysis and its impact on Two Haemolysis based Quantification methods. *J. Sci. Food Agric.* **1990**, *53*, 477−485.

Oleszek, W. Saponin fractions from *Medicago sativa*. Liquid chromatographic separation. Atlas of chromatograms. *J. Chromatogr. Sci.* **1991**, *29*, 128.

Oleszek, W. Alfalfa saponins: structure, biological activity and chemotaxonomy. In *Saponins Used in Food and Agriculture*; Waller, G. R., Yamasaki, K., Eds.; Plenum Press: New York, 1996; pp 155−170.

Oleszek, W.; Jurzysta, M. Isolation chemical characterization and biological activity of alfalfa (*Medicago media* Pers.) root saponins. *Acta Soc. Bot. Pol.* **1986**, *55*, 23−33.

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.* **1990a**, *38*, 1810−1817.

Oleszek, W.; Jurzysta, M.; Price, K. R.; Fenwick, G. R. High performance liquid chrmatography of alfalfa roots saponins. *J. Chromatogr.* **1990b**, *519*, 106−116.

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.

Oleszek, W.; Nowacka, J.; Gee, J. M.; Wortley, G. M.; Johnson, I. T. Effects of some purified alfalfa (*Medicago sativa)* saponins on transmural potential difference in mammalian small intestine. *J. Sci. Food Agric.* **1994**, *65*, 35−39.

Pedersen, M. W. Relative quantity and biological activity of saponins in germinated seeds, roots and foliageof alfalfa. *Crop Sci.* **1975**, *15*, 541−543.

Pedersen, M. W.; Zimmer, D. E.; Anderson, J. O.; McGuire, C. F. A. comparison of saponins from Du-Puits, Ranger and Quinta alfalfas. *Proceedings, 10th International Grassland Congress*; University of Helsinki: Helsinki, 1966; pp 693−698.

Pedersen, M. W.; Zimmer, D. E.; McAllister, D. R.; Anderson, J. O.; Wilding, M. D.; Taylor, G. A.; McGuire, C. F. Comparative Studies of Saponin of Several Alfalfa Varieties Using Chemical and Biochemical Assay. *Crop Sci.* **1967**, *7*, 349−352.

Pedersen, M. W.; Berrang, B.; Wall, M. E.; Davis, K. H., Jr. Modification of spoanin chracteristics of alfalfa by selection. *Crop Sci.* **1973**, *13*, 731−735.

Petersen, D. W. Effects of sterols on the growth of chicks fed high alfalfa diets or a diet containing Quillaja saponin. *J. Nutr.* **1950**, *42*, 597−608.

Plhak, F. Determination of content of Haemolytic saponins in lucerne and its changes during growth and cuts. *Sb. Ved. Pr. Vyzk. Slechtitelsky Ustav Picninarsky Troubku Brna* **1983**, 5−13.

Potter, G. C.; Kummerow, F. A. Chemical similarity and biological activity of the saponins isolated from alfalfa and soybeans. *Science* **1954**, *120*, 224−225.

Price, K. R.; Johnson, I. T.; Fenwick, G. R. The chemistry and bilogical significance of saponins in food and ffeedstuffs. *CRC Crit. Rev. Food Sci. Nutr.* **1987**, *26*, 27−135.

Puszkar, L.; Jastrzebski, A.; Jurzsta, M.; Bialy, Z. Alfalfa saponins as a chance in the integrated hop protection. *Proc. XXXIV Session IOR* **1994**, *2*, 255−259.

Scardavi, A.; Elliot, F. C. A review of saponins in alfalfa and their bioassay utilizing *Trichoderma spp. Bull. Michigan Agric. Exp. Stn., East Lansing* **1967**, *50*, 163.

Shany, S.; Gestetner, B.; Birk, J.; Bondi, A. Effect of lucerne saponins on larval growth anf their detoxification by various sterols. *J. Sci. Food Agric.* **1970a**, *21*, 508−510.

Shany, S.; Birk, Y.; Gestetner, B.; Bondi, A. Preparation, characterization and some properties of saponins from lucerne tops ans roots. *J. Sci. Food. Agric.* **1970b**, *21*, 131−134.

Shany, S.; Gestetner, B.; Birk, Y.; Bondi, A.; Kirson, I. Isolation of hederagenin and the saponin from alfalfa. *Isr. J. Chem.* **1972**, *10*, 881−884.

Shibata, S.; Fujita, M.; Itokawa, H.; Tanaka, O.; Ishii, T. Structure of panaxadiol a sapogenein of ginseng. *Tetrahedron Lett.* **1962**, *10*, 419−422.

Tava, A.; Odoardi, M. Saponins from *Medicago* Sp.: Chemical characterization and biological activity against insects. In *Saponins Used in Food and Agriculture*; Waller, G. R., Yamasaki, K., Eds.; Plenum Press: New York, 1996; pp 97−109.

Tava, A.; Oleszek, W.; Jurzysta, M.; Berardo, N.; Odoardi, M. Alfalfa saponins and sapogenins: isolation and quantification in two different cultivars. *Phytochem. Anal.* **1993**, 269−274.

Timbekova, A. E.; Abubarikov, N. K. Triterpene glycoside of alfalfa. I. Medicoside-G, a novel bisdesmoside from *Medicago sativa*. *Khim. Prir. Soedin.* **1984**, 451−453.

Timbekova, A. E.; Abubarikov, N. K. Triterpene glycosides of alfalfa. II Medicoside C. *Khim. Prir. Soedin.* **1985**, *6*, 805−808.

Timbekova, A. E.; Larin, M. F.; Jagubaev, M. P.; Abubakirov, N. K. Triterpene glycosides of alfalfa. V. Medicaside H. *Khim. Prir. Soedin.* **1989**, *5*, 673−677.

Timbekova, A. E.; Bereschagin, A. L.; Semenov, A. A.; Abubakirov, N. K. Triterpene glycosides of alfalfa. Medicoside L. *Khim. Prir. Soedin.* **1990**, *2*, 221−227.

Timbekova, A. E.; Isaev, M. I.; Abubakirov, N. K. Chemistry and biological activity of triterpenoid glycosides from *Medicago sativa*. In *Saponins Used in Food and Agriculture*; Waller, G. R., Yamasaki, Y., Eds.; Plenum Press: New York, 1996; pp 171−182.

Vacek, L.; Sedlak, B. Comparison of physical, chemical and Biological properties of saponins. *Gunma J. Med. Sci.* **1962**, *11*, 1−6.

West, L. G. Identification of oleanolic acid from saponins present in lucerne (alfalfa) roots. *J. Sci. Food Agric.* **1979**, *30*, 540−542.

Received for review May 9, 1997. Accepted October 13, 1997.[⊗]

JF970389I

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1997.