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SAPONIN CONTENT OF LAHONTAN AND DU PUIITS ALFALFAS

by

George Allan Taylor

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Agronomy

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George Allan Taylor

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INTRODUCTION

Primitive peoples have used saponins for many years to kill fish and as a detergent.

In more recent times saponin has been shown to inhibit the growth of chicks, reduce the egg production of laying hens, and to be a possible factor in bloat of ruminant animals.

Knowledge of relative amounts of saponin in alfalfa would be of value in breeding and utilization research. Preliminary investigations indicated that Du Puits and Lahontan varieties of alfalfa were at opposite ends of the scale in percentage of saponin with Du Puits being the higher. These two varieties have widely divergent areas of origin and use. Du Puits was developed in France and has been found to be of value in Europe and in the eastern United States. Du Puits does well on acid soils but has low resistance to bacterial wilt. Lahontan, however, was developed in Nevada (Dr. O. F. Smith) from Nemas-tan alfalfa and has resistance to the stem nematode, bacterial wilt, and spotted aphid, thus making Lahontan useful in the western United States.

This experiment was designed to study the variation of the percentage of saponin in the alfalfa plant throughout three cutting "periods" (growth periods) and also to study the percentage of saponin in the leaves and stems of Lahontan and Du Puits alfalfas.

REVIEW OF LITERATURE

Occurrence in Plants

Saponins are widely distributed in the plant kingdom. According to Sollman (1948) sapo-glycosides occur in at least 400 plants belonging to 50 different families. He states that saponin is probably formed in the leaves but is found in all parts of the plant. Garner (1961) lists the following families and genera of plants as saponin carriers of toxicological importance: Araceae (Arum), Liliaceae (Narthecium and Paris), Fagaceae (Fagus), Chenopodiaceae (Beta), Caryophyllaceae (many genera), Ranunculaceae (Helleborus), Rosaceae (Quillaja), Leguminosae (Trifolium), Primulaceae (Anagallis and Cyclomen), Solanaceae (Solanum, many species), and Scrophulariaceae (several genera). Research by Maclay and Thompson (1956) indicated that Medicago sativa, Trifolium repens, Medicago lupulina, Lotus corniculatus, and Sorghum sudanense contain saponin. Dhekne and Bhide (1951) found that the pericarp of the seeds of Balanites roxburgii contained 11 percent saponin. Varshney and Shamsuddin (1962) showed that seeds of Albizzia amara contained saponin. The heartwood of Terminalia arjuna was shown to contain saponin by Row and Rao (1962).

Solacolu and Welles (1935) identified saponin in the seed of the following grasses: Brachypodium distachum, Graphiphorum arundinaceum, Aeliopus laevis, Avena pratensis, Aristida capillacea, Gaudinia fragillis, Festuca

alopecurus, Koeleria alpicola, K. cristata, Tricholena rosea, Avena elatior,
Melica altissima, Poa nemoralis, and Panicum aesculentum.

Uses

The foaming of saponin is so conspicuous that many drugs have received suggestive names such as soap bark and soap root. Quillaja (soap bark) and Saponaria (soap root) are used technically for cleansing. Merck's Saponin Purissimum is prepared from Saponaria.

Saponins were used by primitive peoples for cleaning (detergent action) and to poison fish. Dhekne and Bhide (1951) showed that one in 60,000 parts of the alcoholic extract of saponin is lethal to fish. He also stated that crude saponin is lethal to bedbugs.

Sollman (1948) pointed out that Senega saponin is used for chronic coughs. The emulsifying action of Quillaja determines its use for cleansing of articles which are injured by alkalis.

Lindahl et al. (1957) stated that saponins are used as wetting agents in the textile industry in the United States. They are also used in foam-type fire extinguishers. Steroidal saponins are important as drugs and precursors of mammalian hormones.

Factors Affecting Levels in Plants

Results from experiments by Solacolu and Welles (1935) using Phlox spp. and Avena elatior indicated that saponin accumulated in the seeds as the plants reached maturity and was in part used during germination, in metabolism

of growth, and development of sprouts. The rest was distributed throughout the plant until the appearance of the fifth leaf. The disappearance of saponin in the plant corresponded with the time when the assimilation of chlorophyll was completely developed, just as its appearance corresponded with the moment when the reserves took a definitive form in the seed. The above authors indicated that saponin may be a substance that the plant can use as reserve.

Blair, Mitchell, and Silker (1951) reported that free steroidal saponins exist in alfalfa and they decrease as the plant matures. They also found that as the level of nitrogen increased in the soil the sterols (saponin) increased in the alfalfa plant.

The results of Hanson et al. (1963) indicated that the saponin content of alfalfa is influenced by both environmental and genetic factors. This was concluded because the effects of locations, varieties, and cuttings were highly significant. These researchers concluded that with the variation observed among varieties of alfalfa, it would seem reasonable to be able to change the saponin content by breeding. The extent to which this could be done would depend on the genetic variation among individual plants.

A study by Hanson and Kohler (1961) showed that the percentage of saponin in alfalfa was negatively correlated with forage yield and percentage of fiber and correlated positively with percentage of protein.

Chemistry

Saponins are naturally occurring plant glycosides widely distributed in the plant kingdom. They have the property of forming a type of colloidal

solution in water which foams on shaking, like a soap solution, according to Conant and Tishler (1944). The foaming action is due to the fact that the aglycones, or saponinins, are fat-soluble while the carbohydrate portions of the intact saponin molecules are soluble in water.

A pH optimum of 4.5 to 5.0 was found by Mangan (1959) in saponin foams from red clover and alfalfa. Calcium was necessary in saponin solutions for the formation of rigid foams.

A reaction, at room temperature, of a mixture of alfalfa saponins with saturated bromine water was carried on by Bevenue and Williams (1955) in which a white precipitate was obtained. The bromine water cleaved sugars from the alfalfa saponins in the form of sugar polymers.

Lindahl et al. (1957) stated that saponins may be divided into two classes, in one the nucleus or aglycone is steroidal while those of the other classes are triterpenoids.

The basic structure of the triterpenoids (Conant and Tishler, 1944) is shown on page 8. The parent triterpenoids contain 30 carbon atoms and have carbon skeletons which are divisible into six isoprene (isopentane) units (Cook, 1953). Triterpene hydrocarbons have a molecular formula equal to six times that of the isoprene unit (C_5H_8), that is $C_{30}H_{46}$. The more common monohydric triterpenoid alcohols have the formula $C_{30}H_{50}O$, corresponding to hydration of $C_{30}H_{48}$. Florin and Mason (1962) list the soyasapogenols A ($C_{30}H_{50}O_4$), B ($C_{30}H_{50}O_3$), and C ($C_{30}H_{50}O_2$) as being present in Trifolium repens. They also indicate that Glycine max contains soyasapogenol D and Medicago sativa the triterpenoid, medicagenic acid.

Crude saponin isolated from alfalfa is complex in nature and its components are very difficult to separate. Hanson et al. (1963) mentioned that upon hydrolysis mixed alfalfa saponin will yield a mixture of triterpenoid sapogenins (nuclei), sugars, uronic and galacturonic acids. The sugars (differing for the individual saponins) were glucose, arabinose, xylose, galactose, and rhamnose.

The separation of the saponin components is complicated by their similarity of properties. This was advantageous though because it made it possible for Van Atta, Guggolz, and Thompson (1961) to develop a technique which measures the entire complex of saponins in alfalfa.

Potter and Kummerow (1954) mentioned that all genins of alfalfa saponins gave a bright red color on treatment with Liebermann-Burchard reagent typical of triterpenoid nuclei.

Lindahl et al. (1957) stated that saponins are usually insoluble or nearly so in ethyl ether, acetone, petroleum ether, and benzene, but dissolve in polar solvents such as water, alcohol, and pyridine. Some of the legume saponins crystallize from alcohol-water solutions as calcium-magnesium salts. In water solution some saponins form sparingly soluble complexes when treated with an excess of cholesterol. Both these properties have been utilized in studies on the recovery of saponins from legumes.

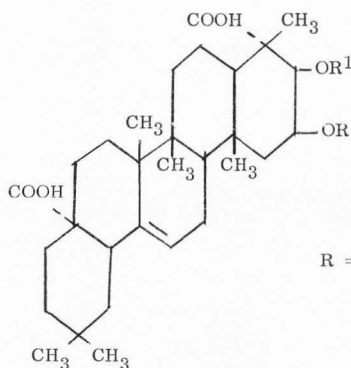
An early investigator, Jacobson (1919), reported an empirical formula of $C_{27}H_{37}NO_{16}$ for alfalfa saponin. Even though the isolated material possessed strong foaming properties, dissolved readily in water, and was lethal to fish, there is doubt that it was saponin, as researchers have found since that saponin does not contain nitrogen.

Evidence of a saponin of alfalfa, yielding in acid hydrolysis of sapogenin, $C_{30}H_{46}O_6$, m. p. 349-350° C, $[\alpha]_D + 111^\circ$, was presented by Walter et al. (1954). A red color was given with the Liebermann-Burchard reagent. Although no direct proof was obtained, the sapogenin appeared to be a triterpene dihydroxy dicarboxylic acid isomeric with costanogenin. Chemical research by Djerassi et al. (1957) revealed a triterpenoid dihydroxy dicarboxylic acid that was a 2β , 3β - dihydroxy - Δ^{12} oleanene - 23, 28 - dioic acid. This confirmed the findings of Walter et al. (1954). The compound was named medicagenic acid. Studies by Potter and Kummerow (1954) showed at least three triterpene genins in dehydrated alfalfa meal. Infrared data from the alfalfa saponins and from a sample of soyasapogenol B indicated that both these materials contain a triterpene nucleus in the aglycone portion.

Paper chromatography of alfalfa saponins by Bevenue and Williams (1955) revealed three spots of an oligosaccharide type of material. Hydrolysis of the three fractions revealed arabinose, xylose, rhamnose, and glucose. Lindahl et al. (1957) obtained a crystalline saponin that appeared identical to a crystalline product obtained from Ladino clover. Upon acid hydrolysis the sugars galactose, glucose, rhamnose, arabinose, and xylose were shown to be present. Glucuronic acid was also found. Chromatography of the sapogenin part resolved it into soyasapogenols B, C, and A. At least six saponins and possibly several more were present in the alfalfa. Seven constituents of alfalfa saponin were found by paper chromatography by Coulson (1958). He also found that the commercial saponin from soapwood bark (Quillaja saponaria) was a mixture of three and possibly four saponins.

Isolation of a new triterpene, $C_{30}H_{46}O_7$, was accomplished by Livingston (1959). It was a trihydroxy, monolactone, monocarboxylic acid and was named lucernic acid.

Medicagenic acid from alfalfa roots was isolated, purified, and structurally identified by Morris, Dye, and Gisler (1961). Chemical constants, elemental analysis, hydroxyl determinations, infrared interpretations, and an NMR study provided evidence for the following structure:



R = H, R¹ = B - D - glucose
for Medicagenic acid

Using paper chromatography, alfalfa saponin was separated into eight components by Lourens and O'Donovan (1961b). Several of these components appeared to be soyasapogenols. More recent chromatographic studies by Coulson and Davies (1962) have shown that there are ten and possibly twelve constituents of alfalfa bulk saponin.

Experiments of Walter *et al.* (1955) revealed at least three saponins in Ladino clover (*Trifolium repens*). Hydrolysis yielded glucose, galactose,

xylose, and rhamnose. Chromatographic separation showed the soyasapogenols B, C, and possibly A to be present. At least six different sapogenins, four of which were as yet unknown, were revealed. It would appear that soyasapogenins may be more common among plants than had hitherto been suspected.

Physiological and Pharmacological Effects

Extracted composite saponins of alfalfa are extremely active and are very toxic depending on the dosage and site of administration. Lindahl et al. (1957) stated that the actions of alfalfa saponins are rather general in nature, having pronounced actions on the cardiovascular, nervous, and digestive systems.

The effect of saponin on the cardiovascular system is rather complex. Saponins possess the ability to hemolyze blood. Sollman (1948) pointed out that the hemolyzing phenomenon depends on the saponin's affinity for the lipoids of the cell envelope and stroma. This is prevented by the addition of cholesterol or other lipoids which combine with saponin. Results of studies conducted by Walter et al. (1955) showed that saponin from Ladino clover (T. repens) did not hemolyze blood at concentrations of 1 percent. Lindahl (1956) stated that there was a rapid and marked drop in the blood pressure of ruminants immediately following an intravenous injection. The most pronounced reaction of saponin on cardiac action was a decrease in the heart rate.

Lindahl (1956) wrote that alfalfa saponin action on the digestive system affects not only the rumen, but also the reticulum, esophagus, and intestine and that animal sensitivity to alfalfa saponin varies. He also stated that consistent

and severe damage to the kidneys and liver was observed in experimental animals. Garner (1961) indicated a number of families of plants that contain steroidal saponins that may cause inflammation of the gastro-intestinal tract.

Results of work conducted by Lindahl et al. (1957) showed that rabbit ileum muscle reacted to a water solution of saponin. There was a progressive diminution of peristalsis, and tonus was at first greatly increased, followed quickly by marked relaxation.

Some researchers claim that saponins increase absorption of sugar and a variety of drugs from the alimentary tract, but the evidence is not conclusive, according to Sollman (1948).

A respiratory inhibitor was isolated from alfalfa meal by Shaw and Jackson (1959). The behavior of the inhibitor during isolation suggested a saponin. In vitro tests on oxygen uptake of rat diaphragm muscle indicated an inhibitory effect on respiration. Experimental work by Coulson and Davies (1962) on the response of rat diaphragm respiration to isolated saponins of alfalfa revealed that at the levels of saponin used no qualitative differences could be shown, but inhibition was found to increase with the amount of saponin added.

According to Sollman (1948) saponin tends to displace other substances or adsorbates from surfaces. In this way saponins tend to alter the permeability of the protoplasmic surfaces of cells and are generally protoplasmic poisons.

Toxic levels of saponin vary with the animal involved. The toxic levels of the composite alfalfa saponins for mature sheep was found by Lindahl et al. (1957) to be 50-60 grams orally or 1 gram intravenously. In research work of

Dollahite, Shaver, and Camp (1962), pregnant rabbits, goats, and cows were given saponin from broomweed (Gutierrezia spp.) intravenously. Other pregnant rabbits were given saponin from Agave lecheguilla and a commercially prepared pharmaceutical grade saponin. It took 5.6 mg of broomweed saponin per kg of body weight daily for 11 days to cause both abortion and death in the pregnant rabbits. For the same period of time 3.6 mg/kg of body weight given to pregnant goats produced the same effect, abortion and death. When 857.0 mg/day of broomweed saponin was given to pregnant cows for 7 days, death resulted. When 358.0 mg/day was given for 26 days the result was an aborted, dead fetus. Intravenous injection of 1.25 mg of Agave saponin per kg of body weight given to pregnant rabbits for 6 days produced abortion. When 17.2 mg of the saponin per kg of weight was given death resulted. When the commercial saponin was used, 6.7 mg/kg of body weight was required to abort rabbits. Using the same material at the rate of 28.6 mg/kg of body weight, death resulted in 3 days.

Factor in Bloat

Saponin as a causal agent of bloat in ruminants has been considered for some time. Jacobson (1919) fed a sheep 19 grams of a saponin preparation with no ill effects. There is doubt this material was saponin, as it contained nitrogen and did not hemolyze blood.

Results of work conducted by Maclay and Thompson (1956) indicated that saponin decreases the surface tension of the ingesta and increases the tendency towards foam. In vitro and in vivo experiments by Lindahl et al.

(1957) support this idea. These experiments showed that alfalfa saponin can contribute to the formation and stabilization of froth of ruminal ingesta.

Using intestinal strips of rabbit and guinea pigs Maclay and Thompson (1956) ran assay tests on alfalfa saponin and a saponin preparation from Baker Chemical Company. When the solution of alfalfa saponin was applied, there was a marked decrease in contractibility of the intestinal strip. The commercial saponin gave no such reaction. The idea was presented that in a cow, alfalfa saponin might contribute to bloat because of rumen paralysis and decreased eructation, hence allowing an accumulation of gases. Lindahl et al. (1957) support this idea as does the work of Walter et al. (1955) on saponin from Ladino clover (T. repens).

Maclay and Thompson (1956) pointed out that alfalfa from farms where bloat occurred contained saponin and alfalfas which did not produce bloat did not contain saponin. Qualitative differences were observed by Coulson and Davies (1962) between the bulk saponins isolated by the cholesterol procedure from dried samples of bloat-producing and nonbloat-producing alfalfa stands. Coulson and Davies (1960), on the other hand, found no qualitative differences between alfalfa saponins isolated from bloat-producing and nonbloat-producing forage on the basis of the response of rat diaphragm respiration. Inhibition increased with the amount of saponin added. They found that bloat-producing alfalfa contained an extra saponin. Cole and Boda (1960) reported that they found a relationship between the saponin content and the bloat potential of Ladino clover, crimson clover, alfalfa, and lespedeza. Feedlot bloat was increased by the addition of soybean meal which contains saponins.

Bloat has been produced experimentally through the administration of saponins. Maclay and Thompson (1956) produced rumen distention in eight of ten sheep after the administration intraruminally of 15 to 25 grams of alfalfa saponin dissolved in 1 pint to 1 quart of water. Gas retention rather than froth seemed to be the cause. Yucca (steroidal) saponin produced no detectable reactions.

Lindahl (1956) and Lindahl et al. (1957) used alfalfa saponins in the experimental production of bloat in sheep. Introduction of 15 to 25 grams of alfalfa saponins intraruminally produced slight to moderate bloat symptoms, whereas 50 grams resulted in severe bloat. Lethal doses appeared to range from 50 to 60 grams. Intravenous injections of 1 to 3 grams of alfalfa saponin resulted in bloat symptoms and death after a period varying from 3 hours to 3 days later.

In similar experiments by Lindahl et al. (1957) using saponin from the yucca plant, bloat symptoms were not produced either by intravenous or intraruminal administration. When 1 gram of Quillaja (Quillaja saponinia) saponin was given intravenously to two sheep, one animal displayed marked bloat symptoms and collapsed. The second animal died within 2 days without a display of bloat symptoms.

Certain saponin-utilizing bacteria isolated from rumina of steers by Gutierrez and Davis (1962) were shown to have the capacity to produce slime when incubated with alfalfa saponins. This slime production was suggested as a significant factor in the bloat syndrome in animals on legume pastures.

Growth Depression Factor in Feed Rations

Experimental work of Lepkovsky et al. (1950) indicated that when alfalfa meal was incorporated in feeds in amounts over 5 percent and fed to chicks a depression in growth occurred. The investigators stated that the growth depressing factor (s) was probably an organic substance. The depressing agent could be removed from alfalfa by repeated extraction with hot water. The depressant was stable to autoclaving.

Evidence that alfalfa meal contains a substance (s) that inhibits the growth of chicks was presented by Peterson (1950b). Materials that depressed the growth of chicks were obtained in a concentrated form by fractionating a hot water extract of alfalfa meal. The feeding of cholesterol almost entirely canceled the growth depressing action of this material. Hemolytic properties, plus observed foaming action, indicated that the material might be saponin. Since alfalfa saponins had not been isolated in pure form it could not be stated categorically that they were responsible for the depression of growth in the chicks.

The effect of saponin on chick growth was investigated by Ackerson et al. (1950) by adding 1 percent commercially prepared saponin to a practical chick ration. The group of chicks on the saponin ration gained an average of 406 grams from hatching to 6 weeks of age with an efficiency of gain of 32 percent. The group on the same ration without saponin gained 459 grams with an efficiency of 34 percent.

The comparative value of sun-cured and dehydrated alfalfa meals and methods of inactivating or eliminating the chick growth depressant factor (s) in them were studied by Kodras, Cooney, and Butts (1951). They found no difference in the rate of growth due to early sun-curing but noticed less growth with normal sun-cured alfalfa. A typical depressing effect of alfalfa meal on chick growth was observed when present in rations at a 20 percent level. Cholesterol was found to counteract the chick growth depressing materials.

Potter and Kummerow (1954) stated that both purified alfalfa and soybean saponins inhibited the growth of chicks while their genins did not. They postulated that part of the growth depressing effect of uncooked soybean meal might be due to the soyasapogenols which upon cooking did not undergo complete hydrolysis to nontoxic genins. These researchers stated that such meals might cause bloat in ruminants and would explain the occurrence of bloat in cattle fed with untoasted solvent-extracted soybean meal as the dehydrating conditions were too mild to hydrolyze alfalfa saponins.

Saponin obtained from the Western Regional Research Laboratory, Albany, California, was used by Heywang and Bird (1954) in diets containing 0, 0.5, 0.10, 0.20, and 0.40 percent saponin. The diets were fed to groups of 10, day-old New Hampshire chicks until they were 6 weeks old. Results showed that saponin in alfalfa inhibited the growth, diet consumption, and efficiency of diet utilization of chicks. The lowest level at which unmistakable growth inhibition occurred was 0.20 percent saponin. The effect was greater at the 0.40 percent level of saponin.

The effect of alfalfa saponin on the performance of chicks and laying hens was reported by Anderson (1957). Chicks were fed saponin isolated from alfalfa at levels from 0.05 to 0.5 percent of the diet. The 0.05 percent level did not affect growth or feed efficiency. A significant decrease in growth and feed efficiency occurred at the 0.1 percent level. As the percentage of saponin in the diet increased from 0.1 to 0.5 percent, the growth depression increased. When 1 percent cholesterol was added, the depression produced by 0.3 percent saponin was overcome. Saponin was fed to laying hens at a 0.3 percent level for 47 days. Egg production dropped immediately following the addition of saponin, but returned to the previous level within 10 days. During the last 5 weeks of the saponin feeding period production was higher than that of three similar pens fed the same diet without saponin. The growth rate of the hens' chicks was not affected by the addition of saponin. On the other hand, studies conducted by Heywang, Thompson, and Kemmerer (1959) revealed that in two experiments pullets fed diets containing 0.40 percent extracted alfalfa saponin laid only 51 percent as many eggs in experiment one as those with the nonsaponin diet and only 43 percent as many eggs in experiment two as those pullets on normal nonsaponin diets. The pullets fed extracted saponin consumed about 29 percent less feed in experiment one and 38 percent less in experiment two than those fed a nonsaponin diet. In two other experiments 0.26 percent saponin was supplied by alfalfa meal in the diet. Those pullets given alfalfa meal laid only 73 percent as many eggs in experiment one and 66 percent as many eggs in experiment two as those fed a diet with no saponin (no alfalfa meal).

A correlation coefficient of +0.844 was obtained by Coulson and Evans (1960) for retardation of weight gain as the percentage of Quillaja saponin was increased in diets of young rats. The addition of cholesterol reversed the action of the Quillaja saponin. Saponin from Gypsophila spp. was similar to Quillaja in its adverse effect on weight gain, but this effect was not reversed by cholesterol. During the experiment it was noticed that the mortality rate of rats on diets containing saponin was much higher than that of rats on diets of no saponin. Results presented by Wilcox and Galloway (1961) verified the foregoing. Two groups of rats were fed 10 percent alfalfa meal in their diets (provided 0.02 percent saponin in the diets). Gains in weight were significantly less for the group of rats fed saponin than for the other groups. When cholesterol was also fed, the gain in weight was somewhat better but not equal to those fed no saponin.

Methods of Isolation of Saponin

Several methods have been devised to isolate saponins from alfalfa. Cholesterol was used by Solacolu and Welles (1935) to isolate saponin. Van Atta, Guggolz, and Thompson (1961) gave an outline of the "cholesteride saponin" recovery method. Yields of "cholestride saponin" are less than the total saponin in alfalfa.

Another process was described by Wall et al. (1952) for the extraction of saponins. Concentrates obtained by alcoholic extraction from alfalfa were defatted with ether and the fat free material extracted with butanol saturated with water. The butanol extracts were evaporated under vacuum until the

saponin started to precipitate. The precipitate was filtered off and suspended in anhydrous acetone. Coulson (1958) and Lourens and O'Donovan (1961a) also kept saponin in suspension in acetone to prevent it from absorbing water which would otherwise result in a dark sticky mass.

Jackson (1960) gave details for the use of a Soxhlet extraction apparatus to obtain aqueous fractions for in vitro biological assays. Van Atta, Guggolz, and Thompson (1961) devised the "carbon-pyridine" saponin recovery and determination method described later in the Methods and Materials section. Van Atta (1962) developed a supplementary treatment of saponin recovered by the carbon-pyridine method that could determine the percentage of purity of the saponin recovered.

MATERIALS AND METHODS

Field Materials

Replicated plots of Lahontan and Du Puits were established on the Greenville Farm in 1961 from the California grown seed lots Lahontan L-3981 and Du Puits W699N.

Sample Preparation

Sampling

A sample of the two varieties was taken from each of the two replications. Each sample was cut by hand and was composed of a number of subsamples taken from different areas in the replication from which the sample came. All weeds and foreign matter were removed at the time of sampling. An effort was made to cut all the samples about 2 inches above the ground level. Sample taking began May 10, 1962, and continued weekly until August 30, 1962, except for periods of time between cuttings necessary for regrowth.

Drying of samples

The green field samples were dried in a steam-heated, forced-air oven at 130-135° F for 24 hours. Thorough, even drying of the plant material was assured by the use of "trays" of one-half inch mesh wire in which the plant material was placed and dried in the oven.

Separation and weighing of stems and leaves

The stems and leaves of the dried plant material were separated by hand, weighed, and placed in paper bags. The separation of leaves from stems was accomplished by "rolling" the dried plant material between the hands while holding the material over a container with a screen covering it, thus preventing most of the stems from falling through. Further separation by hand was necessary to assure a good separation of stems and leaves. The separation of stems and leaves of young succulent plant material was difficult due to the fine stems and petioles.

The percentage of stems and leaves of each sample was determined by weighing the separated material, dividing by the total weight of stems and leaves, and multiplying by 100.

Milling

The leaf and stem samples were ground in a Wiley mill which was cleaned thoroughly between samples with compressed air. The ground samples were immediately returned to the original marked paper bags to avoid mixing the samples.

Determination of Saponins in Alfalfa

Sample drying

An 8-10 gram representative sample was taken from the ground sample and dried in an open moisture can in a vacuum oven at 65^o C for 16 hours and then placed in a desiccator.

Preparation of crude extract

A 4-gram portion of the dried sample was weighed, put into a 250 ml Erlenmeyer flask, 20 ml water was added, mixed, and the mixture let stand for 5 hours. Fifty-five ml of 95 percent ethyl alcohol was added, mixed by swirling, and let stand 16 hours (overnight). Fifteen ml of ethanol and 43 ml of water were added and mixed. This mixture was suction filtered through 9 cm Whatman's number 1 paper after an hour.

Treatment of extract

One gram of activated carbon (discussed later) was added to a 50 ml portion of the crude extract and warmed gently over steam with occasional stirring for 15 minutes. The solution was suction filtered through 9 cm Whatman's number 1 paper precoated with filter aid. The transfer was completed and washed with 100 ml of 50 percent ethanol. The filtrate and washings were evaporated to near dryness over steam to drive off the alcohol. Prolonged or over heating at this point leads to discolored end products and high analytical results.

The evaporation residue was warmed with 20 ml water until solution was complete and 1.5 gram activated carbon was added. The mixture was stirred occasionally while being warmed gently over steam for 5 minutes. The solution was suction filtered through 5.5 cm Whatman's number 50 paper precoated with about 0.5 gram of analytical grade celite deposited from suspension in water. The "filter-cake" was washed with four 20 ml portions of water, followed by two 20 ml portions each 10 percent and 20 percent ethyl alcohol. Both the

filtrate and the washings were discarded.

To elute the saponins from the carbon, the filter was washed with 200 ml of a mixture of pyridine (purified grade) and absolute ethyl alcohol, 3 to 7 (V/V). Throughout filtration, washing, and elution the level of the liquid was not permitted to reach the surface of the filter cake between additions. The pyridine-alcohol eluate was evaporated over steam in a tared dish. The residue and the dish were then vacuum dried for 16 hours at 65° C, cooled in a desiccator, and weighed.

Concerning Pyridine--Saponin Method

The final net weight of saponins was divided by 1.5 grams to give the dry weight percentage of saponin in the sample. The divisor 1.5 represents the grams of dry matter represented in the 50 ml portion of crude extract.

The percentage of saponin recovered from alfalfa samples is higher than the true values. Streak chromatography used by Van Atta and Guggolz (1958) has shown that purity of the saponins recovered is about 75 percent. Tests indicated that this percentage is quite constant. A more recent supplementary chromatographic method for determining saponins in alfalfa, reported by Van Atta (1962), indicated an average of 73.9 percent purity. The supplementary chromatographic method showed the average purity for Lahontan alfalfa was 68.9 percent while that for Du Puits was 68.6 percent.

Carbon Choice and Reactivation

Preliminary trials indicated that the best carbon source was Nuchar C-115-N, obtained from Industrial Chemical Sales, 230 Park Avenue, New York 17, New York.

The carbon was reactivated by an acid digestion process (Van Atta and Guggolz, 1958). In each of four 1500 ml beakers 50 grams of the carbon to be treated was mixed with 1 liter of 1 N HCl. The beakers were immersed in the boiling water of a steam bath for 20 minutes, during which time the mixtures were frequently stirred. The beakers were removed from the bath and the contents allowed to stand until most of the carbon had settled. Most of the warm supernate was decanted onto a 24 cm Whatman's number 1 paper in a Buchner funnel. Complete transfer of the carbon to the funnel was accomplished by water from a wash bottle. The filter cake in the funnel was washed with 3 liters of distilled water, avoiding complete drainage of the cake until all of the water was added. The cake was drained, transferred to a large container and mixed with 6 liters of water. This mixture was filtered as before and the cake washed with distilled water until the washings became free of HCl as shown by test with indicator paper. Again care was exercised in keeping the surface of the cake covered with liquid until all the water was added.

The cake was drained and transferred to an enameled pan. The carbon was reactivated by heating it for 15 hours in a mechanical oven set at 105⁰ C. The carbon was transferred to a quart preserving jar and kept tightly closed except when carbon was being withdrawn for use.

Statistical Analysis

The data were analyzed as a split-plot experiment using the standard analysis of variance, covariance analysis, and Duncan's Multiple Range Test (LSR). A method for expressing the components of variance as percentages was also utilized.

RESULTS AND DISCUSSION

Percentage of Leaves (X)

Dates

The range of the fourteen means for percentage of leaves was from lows of 43.0 percent on June 19 and 46.0 percent on June 14 to highs of 79.7 percent on June 29 and 79.0 percent on August 8 (table 1). The analysis of variance (table 2) showed the variations among the fourteen sampling dates to be highly significant. Dates, as a component of variance, accounted for 91.70 percent of the variation (table 3). Dates could be considered as stages of growth. The percentage of leaves decreased as the plants matured (as sampling dates advanced).

Cuttings

The differences in percentage of leaves among the three cuttings was highly significant. The means for the three cuttings were 52.5 percent, 64.8 percent, and 66.2 percent leaves for the first, second, and third cuttings, respectively (table 1). There was an increase in the percentage of leaves in each successive cutting (figure 1), but the values for the second and third cuttings were similar. There was a decrease in the percentage of leaves as the plants became more mature within each cutting. An examination of any corresponding sampling date within the three cuttings showed an increase in the percentage of

Table 1. Percentage of leaves (X) in Du Puits and Lahontan alfalfas

Dates	Du Puits	Lahontan	Average	LSR ^a
<u>Cutting 1</u>				
5-10	63.0	62.8	62.8	ijk
5-17	55.8	59.1	57.4	efg
5-25	55.0	59.2	57.1	ef
5-31	49.6	53.8	51.7	cd
6-7	48.3	51.0	49.6	bc
6-14	44.7	47.2	46.0	ab
6-19	40.8	45.1	43.0	a
Average	51.0	54.0	52.5	
<u>Cutting 2</u>				
6-29	76.5	82.9	79.7	m
7-10	59.4	59.8	59.6	fghi
7-17	55.3	54.8	55.0	de
Average	63.7	65.8	64.8	
<u>Cutting 3</u>				
8-8	75.9	82.2	79.0	m
8-15	68.5	65.5	67.0	kl
8-22	61.0	59.6	60.3	fghij
8-30	58.6	58.2	58.4	efgh
Average	66.0	66.4	66.2	
Average of cuttings	58.0	60.1	59.0	

^aDuncan's New Multiple Range Test.

Table 2. Analysis of variance of percentage of leaves (X) in Du Puits and Lahontan alfalfas

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F test
Dates	13	6, 013. 23	462. 5562	80. 4751**
(Cuttings)	(2)	(2, 420. 42)	(1, 210. 2100)	211. 2575**
Reps/dates (error a)	14	80. 20	5. 7286	
Varieties	1	57. 01	57. 0100	6. 9301*
Variety x date	13	117. 27	9. 0208	1. 0966
Error (b)	14	115. 17	8. 2264	
Total	55	6, 382. 88		

* Significant at the . 05 level.

** Significant at the . 01 level.

Table 3. Components of variance

Source of variation	Degrees of freedom	EMS
Dates (Cuttings)	13 (2)	$\sigma_b^2 + 2\sigma_{(a)}^2 + 4\sigma_d^2$
Reps/dates error (a)	14	$\sigma_b^2 + 2\sigma_{(a)}^2$
Varieties	1	$\sigma_b^2 + 28\sigma_v^2 + 2\sigma_{vd}^2$
Variety x date	13	$\sigma_b^2 + 2\sigma_{vd}^2$
Error (b)	14	σ_b^2

Percentage of leaves (X)

<u>Component</u>	<u>Estimate</u>	<u>Percent of total variance</u>
σ_b^2	8.2264	6.60
σ_{vd}^2	0.3972	0.32
σ_v^2	1.7139	1.38
$\sigma_{(a)}^2$	-1.2489	0 ^a
σ_d^2	114.2069	91.70

Percentage of saponin in the leaves (Y₁)

<u>Component</u>	<u>Estimate</u>	<u>Percent of total variance</u>
$\sigma_{(b)}^2$	0.0436	2.79
σ_{vd}^2	0.1355	8.67
σ_v^2	0.6648	42.55
$\sigma_{(a)}^2$	-0.0108	0 ^a
σ_d^2	0.7186	45.99

Table 3. Continued

Source of variation	Degrees of freedom	EMS
<u>Percentage of saponin in the stems (Y_2)</u>		
<u>Component</u>	<u>Estimate</u>	<u>Percent of total variance</u>
$\sigma_{(b)}^2$	0.0143	6.63
σ_{vd}^2	0.0148	6.86
σ_v^2	0.0238	11.03
$\sigma_{(a)}^2$	0.0036	1.66
σ_d^2	0.1593	73.82
<u>Percentage of saponin in the aerial portion (Y_3)</u>		
<u>Component</u>	<u>Estimate</u>	<u>Percent of total variance</u>
$\sigma_{(b)}^2$	0.0150	1.38
σ_{vd}^2	0.1025	9.41
σ_v^2	0.2857	26.23
$\sigma_{(a)}^2$	0.0046	0.42
σ_d^2	0.6815	62.56

^aNegative component assumed to estimate zero.

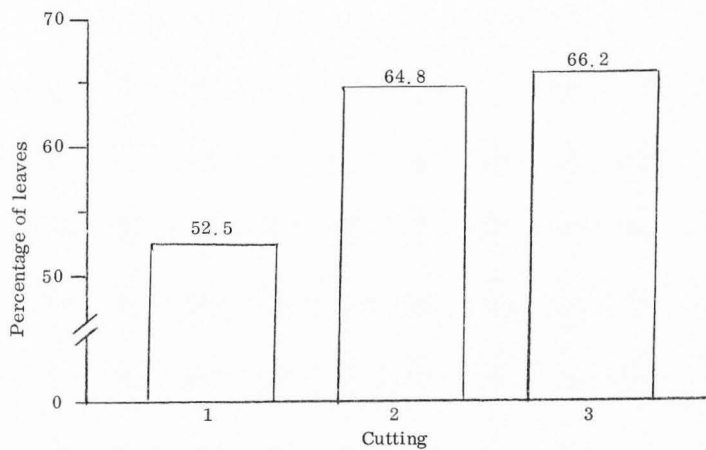


Figure 1. Average percentage of leaves of Lahontan and Du Puits alfalfas

leaves with a progression in cuttings. For example, the second sampling date in each cutting (5-17, 7-10, and 8-15) for Du Puits gave the values 55.8, 59.4, and 68.5 percent leaves for the first, second, and third cuttings, respectively (table 1). The percentage of stems is the reciprocal of the percentage of leaves and therefore need not be discussed.

Varieties

The difference of percentage of leaves between Lahontan and Du Puits varieties of alfalfa was shown to be significant (table 2). The F test gave a value of 6.9301*. Part of the significance of the difference in the percentage of leaves between the varieties Lahontan and Du Puits might be due to the fact Du Puits is more coarse-stemmed than Lahontan and in the separation of stems and leaves more fine stems were included in the Lahontan leaf samples. Hence, a higher overall mean for Lahontan and a significant value for the difference between Lahontan and Du Puits in percentage of leaves. Studies by Hanson et al. (1963) indicated that Lahontan had a higher defoliation score in Eastern United States because of susceptibility to leafspot diseases. In other locations included in this study the mean defoliation score was the same for all varieties. Varieties accounted for only 1.38 percent of the total variance (table 3). The varietal means for percentage of leaves were 60.1 percent for Lahontan and 58.0 percent for Du Puits (table 1 and figure 2). The overall mean percentage of leaves was 59.0.

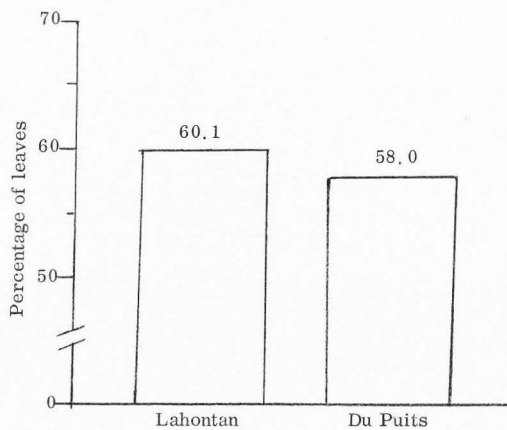


Figure 2. Seasonal average of the percentage of leaves of Lahontan and Du Puits alfalfas

Variety x date

The F test for the variety x date interaction gave no significance (table 2). Only 0.32 percent of the variance can be found in the variety x date interaction (table 3). The component σ^2 error (b) accounted for 6.60 percent of the variance while the σ^2 error (a) term contributed 0.00 percent.

Percentage of Saponin in the Leaves (Y₁)Dates

The range of the means of percentage of saponin in the leaves of Lahontan and Du Puits was from a minimum of 1.79 percent on June 7, first cutting, to a maximum of 4.66 percent on June 29, second cutting (table 4). The mean values of 1.79 percent saponin in the leaves on June 7, 2.56 percent on June 19, 4.66 percent on June 29, 3.82 percent on August 8, and 2.88 percent saponin in the leaves on August 30, all differed significantly from all other means. The analysis of variance showed the differences of percentage of saponin in the leaves of alfalfa to be highly significant when the fourteen sampling dates were examined (table 5). Dates accounted for 45.99 percent of the variation (table 3).

When covariance analysis was utilized, the adjusted mean square for dates was reduced to 55.2 percent of that of the analysis of variance. The F value decreased from 131.0498** in the analysis of variance to 35.8702** in the covariance analysis (tables 5 and 6). Much of the significance, then, of the difference of the percentage of saponin in the leaves on different dates was due to a difference in percentage of leaves on different sampling dates. Even after

Table 4. Average percentage of saponin in the leaves (Y_1) of Du Puits and Lahontan alfalfas

Dates	Du Puits	Lahontan	Average	LSR ^a
<u>Cutting 1</u>				
5-10	2.56	1.76	2.16	bcde
5-17	2.30	1.75	2.02	b
5-25	2.30	1.82	2.06	bc
5-31	2.42	1.76	2.09	bcd
6-7	2.06	1.52	1.79	a
6-14	2.56	1.84	2.20	bcdef
6-19	3.02	2.10	2.56	g
Average	2.46	1.79	2.13	
<u>Cutting 2</u>				
6-29	5.53	3.78	4.66	n
7-10	4.08	2.82	3.45	ijkl
7-17	4.24	2.56	3.40	ijk
Average	4.62	3.05	3.84	
<u>Cutting 3</u>				
8-8	4.60	3.03	3.82	m
8-15	4.46	2.28	3.37	i
8-22	4.20	2.59	3.40	ij
8-30	3.66	2.10	2.88	h
Average	4.23	2.50	3.37	
Average of cuttings	3.43	2.26	2.85	

^aDuncan's New Multiple Range Test.

Table 5. Analysis of variance of percentage of saponin in the leaves (Y_1) of Du Puits and Lahontan alfalfas

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F test
Dates	13	37.65	2.8962	131.0498**
(Cuttings)	(2)	(30.57)	(15.2850)	691.6290**
Reps/dates (error a)	14	0.31	0.0221	
Varieties	1	18.93	18.9300	434.1743**
Variety x date	13	4.09	0.3146	7.2156**
Error (b)	14	0.61	0.0436	
Total	55	61.59		

** Significant at the .01 level.

Table 6. Covariance analysis of percentage of leaves and percentage of saponin in the leaves (XY_1) of Du Puits and Lahontan alfalfas

Source of variation	Degrees of freedom	Sx^2	Sxy	Sy^2	Deviations from regression				Correlation coefficient
					Degrees of freedom	Sum of squares	Mean square	F test	
Dates	13	6,013.23	354.77	37.65					0.7456
(Cuttings)	(2)	(2,420.42)	(259.87)	(30.57)					0.9554
Reps/dates (a)	14	80.20	1.07	0.31	13	0.30	0.0231		0.2146
Varieties	1	57.01	-32.85	18.93					-1.0000
Variety x date	13	117.27	8.97	4.09					0.4096
Error (b)	14	115.17	-1.94	0.61	13	0.58	0.0446		-0.2314
Total	55	6,382.88	330.02	61.59					
Dates + error (a)	27	6,093.43	355.84	37.96	26	17.18			
Difference for testing adjusted date means					13	16.88	1.2985	35.8702**	
Varieties + error (b)	15	172.18	-34.79	19.54	14	12.51			
Difference for testing adjusted variety means					1	11.93	11.9300	267.4887**	
Variety x date + error (b)	27	232.44	7.03	4.70	26	4.49			
Difference for testing adjusted variety x date means					13	3.91	0.3008	6.7444**	

** Significant at the .01 level.

percentage of saponin in the leaves (Y_1) was adjusted for percentage of leaves the variation of percentage of saponin in the leaves on different sampling dates was significantly different ($F = 35.8702^{**}$).

The positive correlation coefficient of +.7456 for dates indicated that as the percentage of leaves increases the percentage of saponin in the leaves also increases (table 6).

Cuttings

The variations of percentage of saponin in the leaves among the three cuttings were highly significant (table 5). The means for the three cuttings were 2.13, 3.84, and 3.37 percent for the first, second, and third cuttings, respectively (table 4 and figure 3). Within the first cutting the percentage of saponin in the leaves decreased as the alfalfa plant matured until about a week before cutting time, when there was a sudden increase in the percentage of saponin in the leaves. The second and third cuttings, however, followed a different pattern. In both the second and third cuttings the percentage of saponin in the leaves of the alfalfa was at a maximum on the first sampling date and decreased steadily to a minimum on the last sampling date. In other words, there was a constant decrease of percentage of saponin in the leaves of alfalfa as the plant matured in both the second and third cuttings.

Varieties

The variation of percentage of saponin in the leaves of Lahontan and Du Puits alfalfas was shown to be significantly different by analysis of variance (table 5). Varieties as a component of variance accounted for 42.55 percent

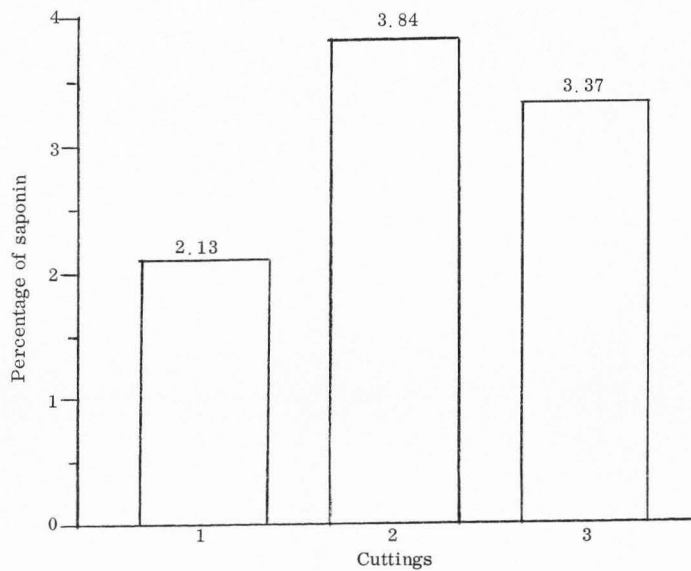


Figure 3. Average percentage of saponin in the leaves (Y_1) of Lahontan and Du Puits alfalfas

of the variation (table 3).

The mean square for varieties was reduced 37.0 percent (tables 5 and 6) in the covariance analysis. The F value was reduced from 434.1743** in the analysis of variance to 267.4887** in the covariance analysis. The difference in percentage of leaves between the two varieties (table 1 and figure 2) would not account for the significant difference of percentage of saponin in the leaves of the two varieties, because the percentage of leaves was higher in Lahontan than Du Puits (table 4 and figure 2). The range of saponin content in the leaves was 1.52 percent to 3.78 percent for Lahontan (table 4), and was 2.06 percent to 5.53 percent for Du Puits. The overall means for Lahontan and Du Puits were 2.26 percent and 3.43 percent, respectively (table 4 and figure 4).

Variety x date

The variation in the variety x date interaction of percentage of saponin in the leaves was highly significant (table 5) in the analysis of variance. The F value was reduced from 7,2156** in the analysis of variance to 6,7444** in the covariance analysis (table 6). These values indicate that the difference in percentage of leaves does account for a part of the significant difference of percentage of saponin in the leaves in the variety x date interaction.

The variety x date interaction as a component of variance accounted for 8.67 percent of the total variation. The component of variance σ^2 error (b) accounted for 2.79 percent of the variance, while the σ^2 error (a) term contributed 0.00 percent (table 3).

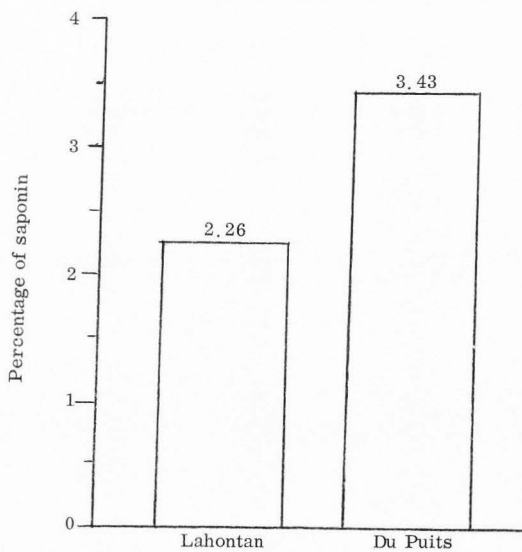


Figure 4. Seasonal average of the percentage of saponin in the leaves (Y_1) of Lahontan and Du Puits alfalfas

Percentage of Saponin in the Stems (Y₂)

Dates

The percentages of saponin in the stems of Du Puits and Lahontan alfalfas among the fourteen sampling dates were significantly different (table 7). As a component of variance, dates accounted for 73.82 percent of the variation (table 3). The means of percentage of saponin in the stems ranged from lows of 0.70 percent on June 7, 0.86 percent on June 19, and 0.90 percent on June 14, to highs of 2.12 percent on June 29 and 1.91 percent on August 8. The overall mean was 1.22 percent saponin in the stems (table 8).

The mean square for dates was reduced from 0.6585 in the analysis of variance to 0.0969 in the covariance analysis, or a reduction of 85.3 percent. The F value was reduced from 30.7710** to 4.3457** (tables 7 and 9). The percentage of leaves, then, accounted for a large portion of the significant difference among dates in the analysis of variance of percentage of saponin in the stems.

The positive correlation coefficient of +.9181 indicated that as the percentage of leaves increased the percentage of saponin in the stems also increased (table 9).

Cuttings

The variations of percentage of saponin in the stems of alfalfa among the three cuttings were highly significant (table 7). The means were 0.94 percent, 1.56 percent, and 1.46 percent saponin for the first, second, and third cuttings,

Table 7. Analysis of variance of percentage of saponin in the stems (Y_2) of Du Puits and Lahontan alfalfas

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F test
Dates	13	8.56	0.6585	30.7710**
(Cuttings)	(2)	(4.41)	(2.2050)	103.0374**
Reps/dates (error a)	14	0.30	0.0214	
Varieties	1	0.71	0.7100	49.6503**
Variety x date	13	0.57	0.0438	3.0629*
Error (b)	14	0.20	0.0143	
Total	55	10.34		

* Significant at the .05 level.

** Significant at the .01 level.

Table 8. Average percentage of saponin in the stems (\bar{Y}_2) of Du Puits and Lahontan alfalfas

Dates	Du Puits	Lahontan	Average	LSR ^a
<u>Cutting 1</u>				
5-10	1.26	1.05	1.16	defghi
5-17	0.92	0.92	0.92	abcd
5-25	0.99	0.94	0.96	bcde
5-31	1.28	0.92	1.10	cdefgh
6-7	0.70	0.70	0.70	a
6-14	0.94	0.85	0.90	abc
6-19	0.87	0.84	0.86	ab
Average	0.99	0.89	0.94	
<u>Cutting 2</u>				
6-29	2.48	1.76	2.12	m
7-10	1.54	1.40	1.47	jk
7-17	1.26	0.94	1.10	cdefg
Average	1.76	1.37	1.56	
<u>Cutting 3</u>				
8-8	2.10	1.72	1.91	m
8-15	1.70	1.29	1.50	jkl
8-22	1.50	1.14	1.32	efghij
8-30	1.12	1.06	1.09	bcdef
Average	1.60	1.30	1.46	
Average of cuttings	1.33	1.11	1.22	

^aDuncan's New Multiple Range Test.

Table 9. Covariance analysis of percentage of leaves and percentage of saponin in the stems (XY_2) of Du Puits and Lahontan alfalfas

Source of variation	Degrees of freedom	Sx^2	Sxy	Sy^2	Deviations from regression			Correlation coefficient
					Degrees of freedom	Sum of squares	Mean square	
Dates	13	6,013.23	208.29	8.56				0.9181
(Cuttings)	(2)	(2,420.42)	(101.14)	(4.41)				0.2044
Reps/dates (a)	14	80.20	2.81	0.30	13	0.29	0.0223	0.5729
Varieties	1	57.01	-6.37	0.71				-1.0000
Variety x date	13	117.27	-0.64	0.57				-0.0783
Error (b)	14	115.17	-1.87	0.20	13	0.17	0.0131	-0.3896
Total	55	6,382.88	202.22	10.34				
Dates + error (a)	27	6,093.43	211.10	8.84	26	1.55		
Difference for testing adjusted date means					13	1.26	0.0969	4.3457**
Varieties + error (b)	15	172.18	-8.24	0.91	14	0.52		
Difference for testing adjusted variety means					1	0.35	0.3500	26.7176**
Variety x date + error (b)	27	232.44	-2.51	0.77	26	0.74		
Difference for testing adjusted variety x date means					13	0.57	0.0438	3.3435*

* Significant at the .05 level.

** Significant at the .01 level.

respectively (table 8 and figure 5). Within the first cutting, after the first sampling date, there was little difference in the percentage of saponin in the stems among dates. There was in general a reduction in saponin content of the stems associated with maturity (table 10).

The percentage of stems is a reciprocal of the percentage of leaves (figure 1) and decreased with each cutting. The percentage of saponin in the stems, however, increased from the first to the second and third cuttings. This is accounted for in the positive correlation coefficient of +.9181 (table 9) that indicated as the percentage of leaves increased (as in cuttings) the percentage of saponin in the stem also increased.

Varieties

The percentage of saponin in the stems of Du Puits and Lahontan alfalfas was significantly different (table 7). Varieties as a component of variance were responsible for 11.03 percent of the variation (table 3). The range of percentage of saponin in the stems was 0.70 percent to 2.48 percent for Du Puits and 0.70 percent to 1.76 percent for Lahontan (table 8). The minimum values were similar for both varieties, while the maximum values were quite different. The mean for Lahontan was 1.11 percent saponin and the mean for Du Puits was 1.33 percent saponin in the stems (figure 6). The overall mean was 1.22 percent saponin in the stems.

The mean square for varieties was reduced from 0.7100 in the analysis of variance to 0.3500 in the covariance analysis, or a reduction of 50.7 percent. The corresponding F value was reduced from 49.6503* to 26.7176** (tables 7

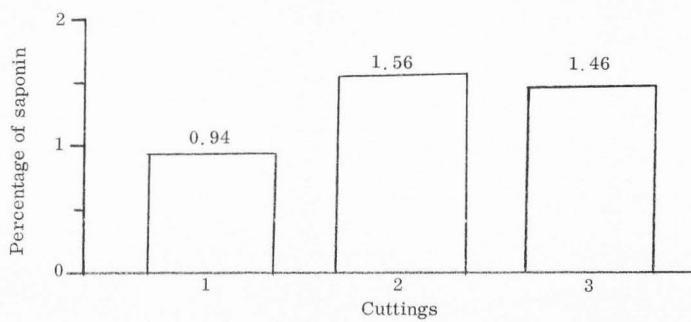


Figure 5. Average percentage of saponin in stems (Y_2) of Lahontan and Du Puits alfalfas

Table 10. Analysis of variance of percentage of saponin in the foliage (Y_3) of Du Puits and Lahontan alfalfas

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F test
Dates	13	35.75	2.7500	113.1687**
(Cuttings)	(2)	(24.68)	(12.3400)	507.8189**
Reps/dates (error a)	14	0.34	0.0243	
Varieties	1	8.22	8.2200	548.0000**
Variety x date	13	2.86	0.2200	14.6667**
Error (b)	14	0.21	0.0150	
Total	55	47.38		

** Significant at the .01 level.

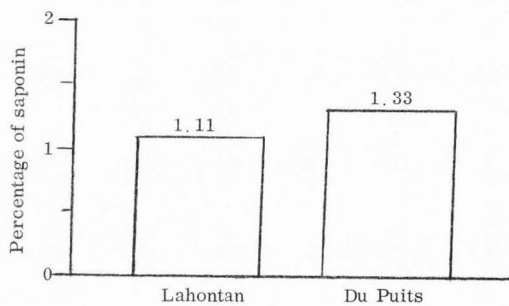


Figure 6. Seasonal mean percentage of saponin in stems (Y_2) of Lahontan and Du Puits alfalfas

and 9). This suggested that even after both the varieties of alfalfa, Lahontan and Du Puits, were adjusted for percentage of leaves, a highly significant difference existed between the varieties in percentage of saponin in the stems.

Variety x date

The variation in the variety x date interaction of percentage of saponin in the stems was significant (table 7). There was no reduction in the mean square for the variety x date interaction of percentage of saponin in the stems. The mean square term was 0.0438** in both the analysis of variance and the covariance analysis (tables 7 and 9). The percentage of leaves did not affect the significance of the variation of the percentage of saponin in the stems in the variety x date interaction.

The variety x date interaction accounted for 6.86 percent of the variance. Of the total variance the component σ^2 error (b) accounted for 6.63 percent of the variation and σ^2 error (a) term accounted for 1.66 percent (table 3).

Percentage of Saponin in the Foliage Portion of the Plant (Y_3)

Dates

The variations of percentage of saponin in the above ground portion of the alfalfa plant among the fourteen sampling dates were highly significant (table 10). The greater part, or 62.56 percent, of the variance is accounted for in dates (table 3). The means ranged from a low of 1.24 percent on June 7 to a high of 4.12 percent saponin in the above ground portion on June 29. The

mean values of 1.24 percent on June 7, 3.40 percent on August 8, and 4.12 percent saponin in the aerial portion of the plant on June 29 all differ significantly from the other means (table 11).

When the source of variation due to dates was adjusted for percentage of leaves in the covariance analysis the mean square for dates was reduced from 2.7500** to 0.6400**, or a reduction of 76.7 percent (tables 10 and 12). The percentage of leaves accounted for a large part of the variance of "dates".

Cuttings

The differences in percentage of saponin in the aerial portion among the three cuttings were highly significant (table 10). The means ranged from 1.24 percent on June 7 to 4.12 percent on June 29 (table 11). Within the first cutting the percentage of saponin in the aerial portion decreased as the alfalfa plant matured until the last two sampling dates, when an increase in percentage of saponin occurred.

Within the second cutting there was a decrease in percentage of saponin in the aerial portion as the alfalfa plant matured or as sampling dates advanced.

In the third cutting a phenomenon similar to the first cutting was observed. The maximum percentage of saponin in the aerial portion of the plant occurred on the first sampling date (table 11). The values decreased as the plant matured except for an increase in saponin on the last sampling date.

The mean percentage of saponin in the above ground portion of the plant for the first cutting was 1.55 percent. The second and third cutting means were higher with 3.04 and 2.97 percent, respectively (table 11 and figure 7).

Table 11. Average percentage of saponin in the foliage (Y_3) of Du Puits and Lahontan alfalfas

Dates	Du Puits	Lahontan	Average	LSR ^a
<u>Cutting 1</u>				
5-10	2.08	1.50	1.79	cdefg
5-17	1.70	1.41	1.56	bc
5-25	1.70	1.46	1.58	bcde
5-31	1.84	1.38	1.61	bcdef
6-7	1.36	1.12	1.24	a
6-14	1.68	1.32	1.50	b
6-19	1.74	1.40	1.57	bcd
Average	1.73	1.37	1.55	
<u>Cutting 2</u>				
6-29	4.81	3.43	4.12	n
7-10	3.05	2.24	2.64	ij
7-17	2.90	1.82	2.36	h
Average	3.59	2.50	3.04	
<u>Cutting 3</u>				
8-8	4.00	2.80	3.40	m
8-15	3.58	1.94	2.76	ijkl
8-22	3.15	2.00	2.58	hi
8-30	2.60	1.66	3.13	l
Average	3.33	2.10	2.97	
Average of cuttings	2.58	1.82	2.27	

^aDuncan's New Multiple Range Test.

Table 12. Covariance analysis of percentage of leaves and percentage of saponin in the foliage (XY₃) of Du Puits and Lahontan alfalfas

Source of variation	Degrees of freedom	Sx ²	Sxy	Sy ²	Deviations from regression				Correlation coefficient
					Degrees of freedom	Sum of squares	Mean square	F test	
Dates	13	6,013.23	406.63	35.75					0.8770
(Cuttings)	(2)	(2,420.42)	(236.86)	(24.68)					0.9691
Reps/dates (a)	14	80.20	3.11	0.34	13	0.22	0.0169		0.5958
Varieties	1	57.01	-21.65	8.22					-1.0000
Variety x date	13	117.27	6.34	2.86					0.3462
Error (b)	14	115.17	1.03	0.21	13	0.20	0.0154		
Total	55	6,382.88	395.46	47.38					
Dates + error (a)	27	6,093.43	409.74	36.09	26	8.54			
Difference for testing adjusted date means					13	8.32	0.6400	37.8698**	
Varieties + error (b)	15	172.18	-20.62	8.43	14	5.96			
Difference for testing adjusted variety means					1	5.76	5.7600	374.0260**	
Variety x date + error (b)	27	232.44	7.37	3.07	26	2.84			
Difference for testing adjusted variety x date means					13	2.64	0.2031	13.1883**	

** Significant at the .01 level.

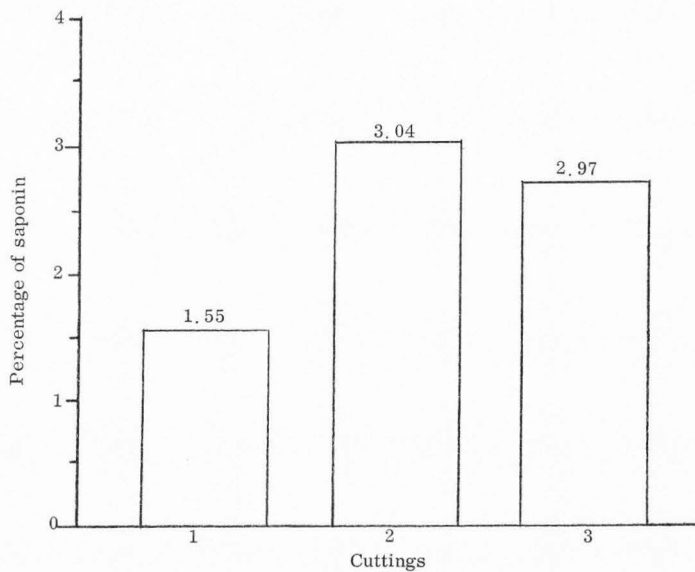


Figure 7. Mean percentage of saponin in foliage (Y_3) of Lahontan and Du Puits alfalfas

These results are confirmed by the work of Hanson et al. (1963) with a number of different alfalfa varieties. They noted that the first cutting was lower in saponin content than the other two cuttings at six of eight locations. The second and third cuttings were approximately equal in percentage of saponin in the above ground portion of the alfalfa plant. Other results by the same researchers indicated the high yield that occurred in the first cutting was accounted for by optimum moisture and other environmental conditions. Since saponin was low in the first cutting, it appeared that environmental factors that favored high yield also favored low saponin. This was opposite the genetic situation, where the lowest yielding varieties were also lowest in saponins.

Varieties

A highly significant difference of percentage of saponin in the aerial portion of the plant occurred between the varieties Lahontan and Du Puits (table 10). Varieties, as a component of variance, accounted for 26.23 percent of the total variation. In work with four "core" varieties at eight locations Hanson et al. (1963) found that varieties as a source of variation contributed 16.4 percent to the variance. The range of percentage of saponin in the aerial portion was from 1.12 percent on June 7 to 3.43 percent on June 29 for Lahontan, and 1.36 percent on June 7 to 4.81 percent on June 29 for Du Puits (table 11). The overall mean for Du Puits was 2.58 percent, this being somewhat higher than Lahontan at 1.82 percent saponin in the aerial portion (figure 8). The mean (over both varieties) was 2.27 percent saponin (table 4). These findings are in accord with those of Hanson et al. (1963) in which Lahontan was

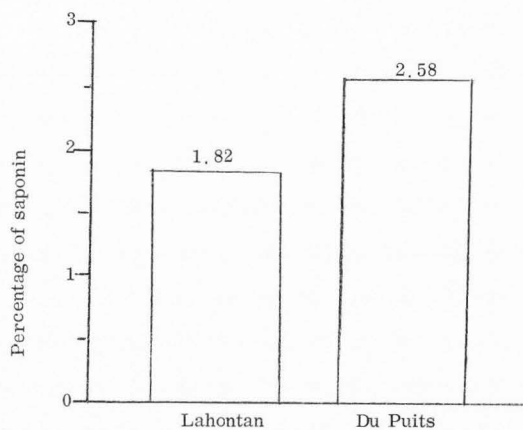


Figure 8. Seasonal mean percentage of saponin in foliage (Y₃) of Lahontan and Du Puits alfalfas

the lowest ranked variety at all eight locations. Du Puits was included at only three locations but was highest in percentage of saponin (aerial portion) at each of these three locations. Findings by these workers further indicated that the genetic difference in saponin content between Lahontan and other varieties of alfalfa apparently was not accounted for by differences in defoliation.

When "varieties" was adjusted to a common leaf percentage the mean square was reduced 29.9 percent, or from 8.2200 in the analysis of variance to 5.7600 in the covariance analysis. The F value decreased from 548.0000** to 374.0260** (tables 10 and 12). The percentage of leaves, then, did not account for a very large portion of the significant difference between varieties in the percentage of saponin in the above ground portion of the plant.

As the leaves of the two alfalfa varieties contained a much higher percentage of saponin than the stems (tables 4 and 8) the leaves influenced the percentage of saponin in the aerial portion of the alfalfa more than the stems.

Variety x date

The variation of percentage of saponin in the aerial portion in the variety x date interaction was highly significant (table 10). The variety x date interaction accounted for 9.41 percent of the variance (table 3).

Through the use of a three-dimensional graph (figure 9) some of the larger variety x date interactions were observed. The uneven ratio of the change of the two varieties in percentage of saponin in the aerial portion at given dates constitutes the variety x date interaction. The percentage of saponin in the aerial portion of Du Puits decreased from 2.08 percent on

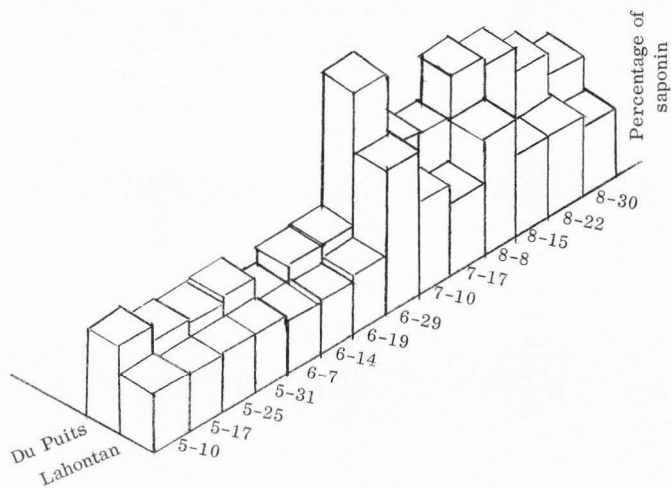


Figure 9. Three-dimensional graph of variety x date interaction of Lahontan and Du Puits alfalfas

May 10 to 1.70 percent on May 17. The values for Lahontan on the corresponding dates remained almost constant (table 4). On July 10 Du Puits alfalfa contained 3.05 percent saponin in the above ground part of the plant. This decreased slightly to 2.90 percent on the next sampling date, July 17. Lahontan, however, on the same dates decreased a larger amount, from 2.24 percent to 1.82 percent saponin in the aerial portion. On the consecutive sampling dates, August 15 and August 22, Lahontan contained 1.94 percent and 2.00 percent saponin in the aerial portion. Du Puits decreased sharply from 3.58 percent on August 15 to 3.15 percent saponin on August 22.

The same interactions were observed (figure 10) by the areas of non-parallelism of the lines between the dates mentioned in the foregoing paragraph.

The component σ^2 error (a) accounted for 0.42 percent of the total variance. The σ^2 error (b) term likewise accounted for 1.38 percent of the variance (table 3).

The percentage of leaves and the percentage of saponin in the aerial portion are correlated in a positive manner (figure 11). Of the total variation of percentage of saponin in the whole plant, 70 percent is associated with the variation in the percentage of leaves.

Effect of Sprinkler Irrigation

In order to see if rain might affect the percentage of saponin in alfalfa, a small experiment was devised in which a sprinkler was placed between adjoining replications of Lahontan and Du Puits. The alfalfa was sprinkled for 10 hours, samples were taken in the sprinkled area and outside the sprinkled

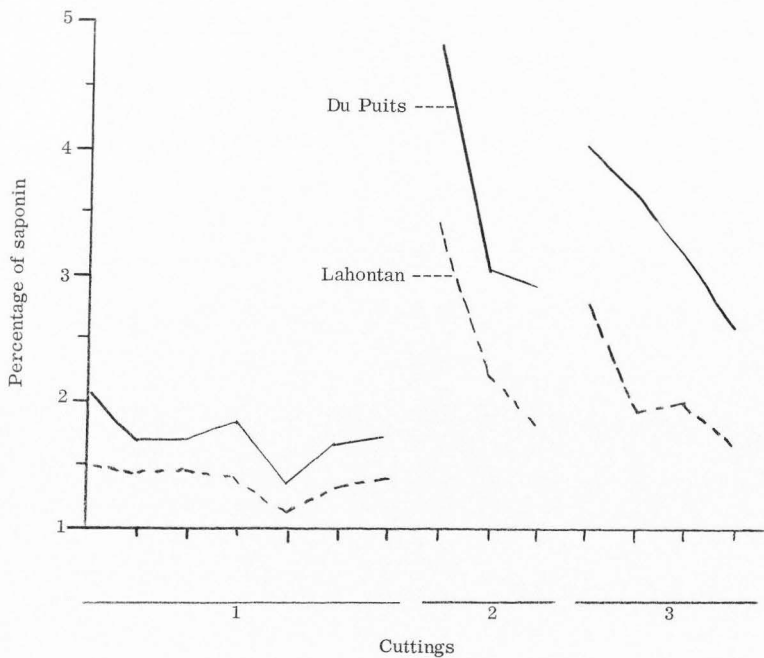


Figure 10. Percentage of saponin in foliage (Y₂) of Lahontan and Du Puits alfalfas

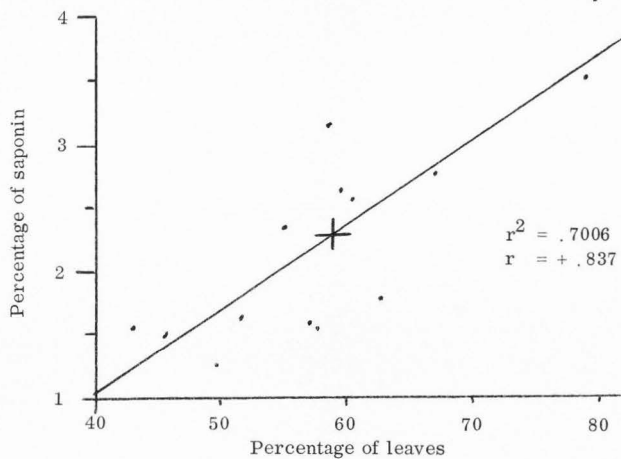


Figure 11. Regression of percentage of leaves and percentage of saponin in foliage (Y_3) of Lahontan and Du Puits alfalfas

area. Results showed that Du Puits decreased in percentage of saponin in the aerial portion from 3.72 percent in the unsprinkled, to 3.26 percent in the sprinkled area, or a loss of 12.36 percent. The percentage of saponin in the aerial part of the plant for Lahontan was decreased from 2.31 percent in the unsprinkled, to 2.02 percent in the sprinkled area, or a 12.55 percent loss in saponin (table 13 and figure 12).

Other more detailed experiments need to be conducted to study environmental effects on the saponin content of alfalfa. Experiments including the root portion and seed would help to establish an understanding of the plant-saponin relationship in alfalfa.

A saponin analysis on a sample of Du Puits alfalfa seed revealed that 1.25 percent saponin was contained in the seed.

Table 13. Effect of sprinkler irrigation^a on percentage of saponin in the foliage of Du Puits and Lahontan alfalfas

Variety	Percent saponin		Percent saponin lost
	Not sprinkled	Sprinkled	
Du Puits	3.72	3.26	12.36
Lahontan	2.31	2.02	12.55

^aSprinkled 10 hours.

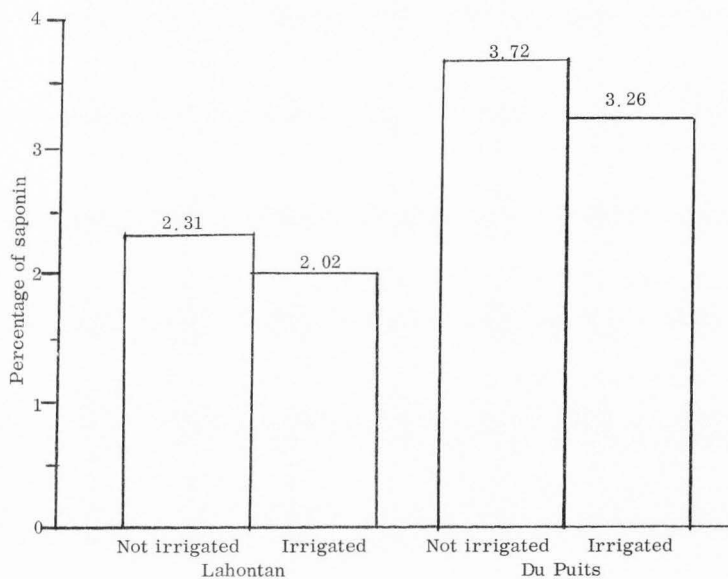


Figure 12. Effect of sprinkler irrigation, sprinkled 10 hours, on the percentage of saponin in the foliage of Lahontan and Du Puits alfalfas

SUMMARY AND CONCLUSIONS

Data from fourteen sampling dates were analyzed in a study of the saponin content of the leaves, stems, and aerial portion of Du Puits and Lahontan alfalfas.

Stages of Growth

Variations due to different stages of growth were highly significant when percentage of leaves, percentage of saponin in the leaves, stems, and in the aerial portion were considered. Covariance analysis of the percentage of saponin in the leaves, stems, and aerial portions of Du Puits and Lahontan alfalfas indicated that the variation of percentage of leaves was responsible for a large segment of the difference due to stages of growth shown by the analysis of variance.

"Cuttings" was a highly significant source of variation for percentage of leaves and percentage of saponin in stems, leaves, and aerial portion.

Effect of Varieties

The difference between varieties was significant at the .05 level for percentage of leaves, but highly significant for percentage of saponin in the leaves, stems, and aerial portions of Du Puits and Lahontan alfalfas.

An adjustment to a common percentage of leaves (covariance analysis) showed that varieties accounted for an appreciable part of the difference in percentage of saponin in the leaves, stems, and aerial portion found in the analysis of variance.

Effect of Variety x Date Interaction

The variety x date interaction as a source of variation was not significant for percentage of leaves. Significance was shown at the .05 level for the percentage of saponin in the stems of Du Puits and Lahontan alfalfas in the variety x date interaction. Highly significant values for the variety x date interaction occurred in the analysis of variance for the percentage of saponin in the leaves and in the aerial portion of the plants. When an adjustment to a common percentage of leaves was made the same pattern occurred. Significance at the .05 level was shown for the percentage of leaves and the percentage of saponin in the stems, while significance at the .01 level was indicated for both the percentage of leaves and percentage of saponin in the leaves and aerial portion of Du Puits and Lahontan alfalfas.

The percentage of leaves did not account for a substantial part of the significance of the variety x date interaction indicated by the analysis of variance.

General

The percentage of saponin in the foliage of Lahontan and Du Puits was decreased 12 percent when sprinkler irrigation was used. Rain might produce

a similar change.

If a particular level of saponin was desired in an alfalfa product, selection for variety, cutting, and stages of growth within cuttings should be considered.

Qualitative as well as quantitative differences of percentage of saponin in alfalfa should be considered as a possible important factor in further saponin studies.

LITERATURE CITED

- Ackerson, C. W., R. L. Borchers, J. E. Temper, and F. E. Mussehl. 1950. Growth inhibition of saponin in alfalfa feeding. Nebraska Agricultural Experiment Station 63rd Annual Report, p. 73.
- Allen, R. S., R. E. Worthington, N. R. Gould, N. L. Jacobsen, and A. E. Freeman. 1961. Forage constituents: diurnal variation in composition of alfalfa. *Agricultural and Food Chemistry* 9 (5): 406.
- Anderson, J. O. 1957. Effect of alfalfa saponins on the performance of chicks and laying hens. *Poultry Science* 34 (4): 873-876.
- Bevenue, Arthur, and K. T. Williams. 1955. Reaction of bromine water with alfalfa saponins. *Science* 121:106.
- Blair, E. H., H. L. Mitchell, and R. E. Silker. 1951. The sterols of alfalfa and certain cereal grasses. *Plant Physiology* 26: 337-342.
- Cole, H. H., and J. M. Boda. 1960. Continued progress toward controlling bloat. *Journal of Dairy Science* 43 (11): 1585-1614.
- Conant, J. B., and M. Tishler. 1944. *The chemistry of organic compounds*. Macmillan Company, New York.
- Cook, J. W. 1953. *Progress in organic chemistry*. II. Academic Press, New York and London.
- Coulson, C. B. 1958. Triterpenoid saponins from lucerne and other species. *Journal of the Science of Food and Agriculture* 9: 281-288.
- Coulson, C. B., and T. Davies. 1960. Lucerne saponin fractionation and bloat. *Nature* 188: 947-948.
- Coulson, C. B., and T. Davies. 1962. Fractionation and pharmacological properties of lucerne saponins. The possible relation of these to bloat. *Journal of the Science of Food and Agriculture* 13: 53-57.
- Coulson, C. B., and R. A. Evans. 1960. The effect of saponin, sterols and linoleic acid on the weight increase of growing rats. *British Journal of Nutrition* 14: 121.

- Dhekne, V. V., and B. V. Bhide. 1951. Chemical investigation of the seeds of *Balanites roxburgii*, Planch (hingora seeds), Part I. Journal of the Indian Chemical Society 28: 588-590.
- Djerassi, Carl, D. B. Thomas, A. C. Livingston, and C. R. Thompson. 1957. Terpenoids. XXI. The structure and stereochemistry of medicagenic acid. American Chemistry Society Journal 79: 5292-5297.
- Dollahite, J. W., T. Shaver, and B. J. Camp. 1962. Injected saponins as abortifacients. American Journal of Veterinary Research 23 (97): 1261-1263.
- Florin, M., and H. S. Mason. 1962. Constituents of life--part A. Comparative biochemistry. Vol. III. Chapters 10 and 11. Academic Press, New York and London.
- Garner, R. J. 1961. Veterinary toxicology. Second edition. Williams and Wilkins Company, Baltimore, Maryland.
- Gutierrez, J., and R. E. Davis. 1962. Isolation of saponin-digesting bacteria from the rumen of bloating cattle on Ladino clover pasture. Journal of American Science 21 (4): 819-823.
- Gutierrez, J. R., R. E. Davis, and T. L. Lindahl. 1959. Characteristics of saponin-utilizing bacteria from the rumen of cattle. Applied Microbiology 7 (5): 304-308.
- Hanson, C. H., and G. O. Kohler. 1961. Progress report on a study of cultural factors related to estogen and saponin content of alfalfa. Seventh Technical Alfalfa Conference, Albany, California, 1961.
- Hanson, C. H., G. O. Kohler, J. W. Dudley, E. L. Sorenson, G. R. Van Atta, K. W. Taylor, M. W. Pedersen, H. L. Carnahan, C. P. Wilsie, W. R. Kehr, C. C. Lowe, E. H. Stanford, and J. A. Yungen. 1963. Saponin content of alfalfa as related to location, cutting variety, and other variables. Agriculture Research Service, United States Department of Agriculture Publication, pp. 34-44.
- Heywang, B. W., and H. R. Bird. 1954. The effect of alfalfa saponin on the growth, diet consumption and efficiency of diet utilization of chicks. Poultry Science 33: 239-241.
- Heywang, Burt W., C. R. Thompson, and A. R. Kemmerer. 1959. Effect of alfalfa saponins on laying chicks. Poultry Science 38 (4): 968-971.

- Jackson, H. D. 1960. Fractionation procedure for Jackson's method of saponin extraction. Letter from Department of Biochemistry, Purdue University, Lafayette, Indiana.
- Jacobson, C. A. 1919. Alfalfa saponin. Alfalfa investigation VII. American Chemical Society Journal 41:640-648.
- Kodras, R., W. T. Cooney, and J. S. Butts. 1951. Chick growth depressing factor in suncured and dehydrated alfalfa meals. Poultry Science 30: 280-292.
- Lepkovsky, S., W. Shaeleff, D. Peterson, and R. Perry. 1950. Alfalfa inhibitor in chick rations. Poultry Science 29: 208-213.
- Lindahl, I. L. 1955. The pharmacology of some of the alfalfa saponins. United States Department of Agriculture Publication.
- Lindahl, I. L. 1956. Effect of saponin on froth formation of ruminal contents. United States Department of Agriculture, Report on the Third Conference on Rumen Function.
- Lindahl, I. L., R. E. Davis, R. T. Tertell, G. E. Whitmore, W. T. Shalkop, R. W. Dougherty, C. R. Thompson, G. R. Van Atta, E. M. Bickoff, E. D. Walter, A. G. Livingston, J. Guggolz, R. H. Wilson, M. B. Sideman, and F. De Eds. 1957. Alfalfa saponins. United States Department of Agriculture Technical Bulletin 1161.
- Livingston, A. L. 1959. Lucernic acid = a new triterpene from alfalfa. Journal of Organic Chemistry 24: 1567-1568.
- Lourens, W. A., and M. B. O'Donovan. 1961a. Studies on the lucerne saponins. I. The isolation of saponins. South African Journal of Agricultural Science 4(2):151-156.
- Lourens, W. A., and M. B. O'Donovan. 1961b. Studies on the lucerne saponins. II. The isolation of triterpene genins from lucerne saponin. South African Journal of Agricultural Science 4(3):293-300.
- Maclay, W. D., and C. R. Thompson. 1956. A review of bloat in ruminants. Report of committee on Animal Health of the Agricultural Board.
- Mangan, J. L. 1959. The foaming properties of proteins, saponins, and rumen liquor. New Zealand Journal of Agricultural Research 2(1): 47-51.

- Mangan, J. L., and A. T. Johns. 1957. A comparison of the chemical composition and foaming properties of extracts of bloating and nonbloating clover. *New Zealand Journal of Science and Technology* 38:956-965.
- Mertz, E. T., and J. W. Porter. 1950. *Plant and animal biochemistry*. Burgess Publishing Co., Minneapolis, Minnesota.
- Morris, R. J., W. B. Dye, and P. S. Gisler. 1961. Isolation, purification, and structural identity of an alfalfa root saponin. *Journal of Organic Chemistry* 26:1241.
- Peterson, D. W. 1950a. Effect of sterols on the growth of chicks fed high alfalfa diets or a diet containing Quillaja saponin. *Journal of Nutrition* 42 (4): 597.
- Peterson, D. W. 1950b. Some properties of a factor in alfalfa meal causing depression of growth in chicks. *Journal of Biological Chemistry* 183: 647-653.
- Potter, G. C., and F. A. Kummerow. 1954. Chemical similarity and biological activity of the saponins isolated from alfalfa and soybeans. *Science* 120:224-225.
- Reilstotter, L., and F. Schipke. 1951. Quantitative determination of saponins by measurements of surface tension. *Arch. der Pharmazie und Berichte der Deutschen Pharmazentischen Gesellschaft* 284 (54): 101-106.
- Row, L. Ramachandra, and G. S. R. Subba Rao. 1962. Chemistry of Terminalia species. *Journal of Indian Chemical Society* 39 (2): 89-92.
- Ruyssen, R. 1947. The determination of saponins by means of the hemolytic index. *Journal de Pharmacie de Belgique* 2:262-283.
- Shaw, R. A., and H. D. Jackson. 1957. Fractions from Ladino clover which inhibit biological systems. *Journal of Animal Science* 16 (3): 711-718.
- Shaw, R. A., and H. D. Jackson. 1959. Isolation of a respirator inhibitor from alfalfa. *Archives of Biochemistry and Biophysics* 84:405-410.
- Solacolu, T., and Ec. Welles. 1935. Changes in saponin of several species of grass during germination and growth. *Compte Rendu de la Societe Biologie* 120:662-664.
- Sollman, T. H. 1948. *A manual of pharmacology and its applications to therapeutics and toxicology*. Edition Seven. W. B. Saunders Company, Philadelphia, Pennsylvania.

- Van Atta, G. R. 1962. Supplementary chromatographic method for determining saponins in alfalfa. *Agricultural and Food Chemistry* 10 (6): 519-520.
- Van Atta, G. R., and J. Guggolz. 1958. Forage constituents: detection of saponins and sapogenins on paper chromatograms by Liebermann-Burchard reagent. *Agricultural and Food Chemistry* 6 (11): 849.
- Van Atta, G. R., J. Guggolz, and C. R. Thompson. 1961. Determination of saponins in alfalfa. *Agricultural and Food Chemistry* 9 (1): 77.
- Van Duuren, A. J. 1962. Thin layer chromatography of sugar beet saponins. *Journal of American Society of Sugar Beet Technology* 12 (1): 57-63.
- Varshney, I. P., and K. M. Shamsuddin. 1962. Saponins and sapogenins. *Journal of Scientific and Industrial Research* 21 (7): 347.
- Wall, E. D., M. Krider, E. S. Rothman, and C. R. Eddy. 1952. Steroidal sapogenins. *Journal of Biological Chemistry* 198: 533-543.
- Walter, E. D., E. M. Bickoff, C. R. Thompson, C. H. Robinson, and C. Djerassi. 1954. Alfalfa saponin. *Journal of American Chemical Society* 76: 2271.
- Walter, E. D., G. R. Van Atta, C. R. Thompson, and W. D. Maclay. 1955. Saponin from Ladino clover (*Trifolium repens*). *Journal of American Chemical Society* 77 (4): 4936-4937.
- Wilcox, E. B., and L. S. Galloway. 1961. Serum and liver cholesterol, total lipids and lipid phosphorus levels of rats under various dietary regimens. *American Journal of Clinical Nutrition* 9: 236-243.
- Wilson, R. H., M. B. Sideman, and F. De Eds. 1956. Some pharmacological effects of saponins of alfalfa and Ladino clover. *Federation Proceedings* 15: 1627.