licate by the procedure of Benson and Patterson (1965) and the results were statistically evaluated (Table IV). Only arginine and glycine showed statistically significant variation at the 5% level and the variation was associated with the heavily damaged kernels. When a mold is present, the appearance of another protein could be expected; however, none was detected in a quantity sufficient to alter the protein composition. Several unusual amino acid peaks were noted in the heavily damaged kernels as might be expected with mold or bacteria present, but their quantity was small. Only two amino acids were significantly affected, and then only in heavily damaged kernels. Blighted corn had essentially the same protein composition as the nonmoldy corn tested.

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Titrimetric Method for Determination of Medicagenic Acid in Alfalfa (*Medicago sativa*)[1]

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A titrimetric method for the quantitative determination of medicagenic acid (a biologically active sapogenin present in alfalfa saponins) in the presence of other nonactive sapogenins in the acid hydrolysates of alfalfa tops and roots has been elaborated. The amount of medicagenic acid containing saponins can be derived from the content of medicagenic acid by means of a conversion factor. The content of these saponins in alfalfa tops and roots is 0.11 and 0.96%, respectively, thus accounting for the greater toxicity of the latter toward various organisms.

During recent years there has been interest in determining the relationship of saponin content in alfalfa (*Medicago sativa*) to a number of adverse physiological effects. At least ten different saponins are present in alfalfa. Soyasapogenols A, B, C, D, and E and medicagenic acid were identified in the aglycone moiety and glucose, galactose, arabinose, xylose, rhamnose, and glucuronic acid in the carbohydrate moiety (for review see Birk, 1969).

It has been shown recently that several biological activities of alfalfa saponins, such as hemolysis and larval and fungal growth inhibition, are exerted only by those saponins which contain medicagenic acid as their aglycone (Gestetner *et al.*, 1971a,b; Shany *et al.*, 1970a). The need for determination of the amounts of medicagenic acid in the presence of the other aglycones is therefore evident. Since medicagenic acid is a triterpenoid acid with two carboxyl groups Djerassi *et al.*, 1957), which are absent from the other alfalfa sapogenins, a titrimetric method has been elaborated for this purpose.

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[1] This is Part VIII of a series on "Alfalfa Saponins;" Part VII appeared in *Israel J. Chem.* (1972).

MATERIALS AND METHODS

Quantitative Determination of Medicagenic Acid. A mixture of alfalfa sapogenins isolated from purified saponin extracts (SE) prepared from alfalfa (*Medicago sativa*) tops flour, Hairy Peruvian variety, or from its roots, according to the method of Shany *et al.* (1970b), was used. A sample of 30 mg of SE was dissolved in 30 ml of 1 *N* H_2SO_4 in dioxane–water (1 : 3) and boiled under reflux for 12 hr. This period ensures complete hydrolysis of alfalfa saponins (Shany *et al.*, 1970b). The liberated sapogenins were extracted with four portions of ether (50 ml each); the combined extracts were washed with water for removal of residual H_2SO_4 and concentrated to dryness in a rotary vacuum evaporator.

The sapogenin mixture was dissolved in 50 ml of absolute methanol. Aliquots of 1 ml, to which two drops of thymolphthalein has been added, were titrated with 0.1 *N* methanolic KOH using an Agla micrometric syringe. The color transition at pH 10.0 marked completion of the titration of both carboxyl groups. Prior to and during the titration, nitrogen was introduced through the solution in order to remove carbon dioxide and as a means of stirring. The calculation of the amount of medicagenic acid was based on a molecular weight of 502 (Djerassi *et al.*, 1957).

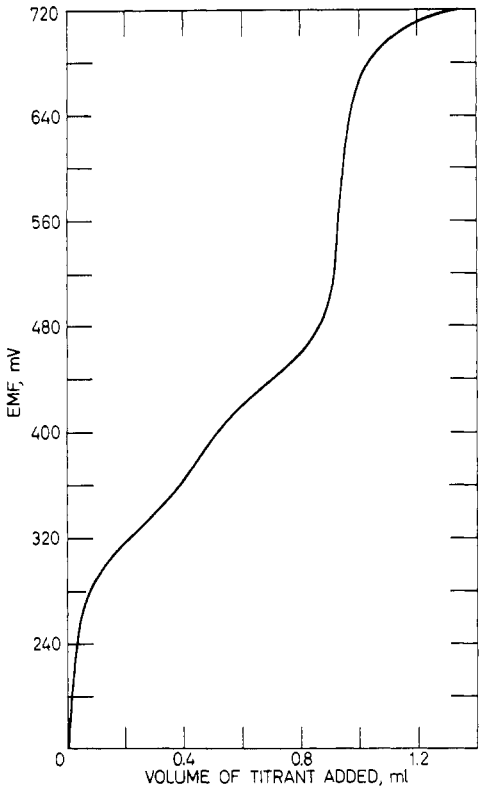

Figure 1. Titration curve of medicagenic acid obtained by automatic potentiometric titration. Solvent: dimethyl formamide–*tert*-butanol (4:6); titrant: 0.05 *N* tetrabutyl ammonium hydroxide

Table I. Medicagenic Acid and Medicagenic Acid Containing Saponin Content of Alfalfa Tops and Roots

Material examined	Medicagenic acid content, mg/g	Medicagenic acid–sugar ratio[a]	% saponin
Alfalfa tops	0.26	1:3.08	0.11
Alfalfa roots	4.48	1:1.14	0.96

[a] See Gestetner *et al.* (1971a).

Preparation of a Titration Curve for Medicagenic Acid. For this purpose medicagenic acid was isolated and purified as described by Morris *et al.* (1961). Titration curves of the carboxyl groups were determined by automatic potentiometric titrations in a nonaqueous medium (Ehrlich-Rogozinsky, 1971), using dimethyl formamide–*tert*-butanol (4:6) as solvent. The titrant was 0.05 *N* tetrabutyl ammonium hydroxide.

Determination of the Content of Medicagenic Acid in Alfalfa Flour. 10 g of alfalfa tops flour were suspended in 200 ml of 50% ethanol and the saponins were extracted at 60° with constant stirring overnight. The extract was filtered on a Büchner funnel and the residue was washed with 100-ml portions of hot 50% ethanol until no saponins could be detected by tlc in the washings. After 5–6 washings only traces of saponins could be detected by tlc in the 50% ethanolic extract of the residual cake. The combined washings were concentrated to an aqueous suspension, 2 g of cholesterol was added, and the mixture was boiled for 60 min for precipitation of medicagenic acid-containing saponins (Gestetner *et al.*, 1971a; Walter *et al.*, 1954). After cooling, the mixture was filtered on a Büchner funnel and the precipitate was washed with 400 ml of water and 400 ml of benzene for removal of nonprecipitated saponins and excess cholesterol, respectively.

Cholesterol-bound alfalfa saponins were liberated from the precipitate by washing successively with 500 ml of acetone, 500 ml of hot 96% ethanol, and 500 ml of hot 50% ethanol. The washings were combined and concentrated to an aqueous solution. Sulfuric acid was added to produce a concentration of 1 *N* and acid hydrolysis of the saponins was carried out under reflux for 12 hr. The sapogenins were extracted from the hydrolyzate with four portions of ether (100 ml each). The combined extracts were washed with water, concentrated to dryness, and stored in a desiccator over P_2O_5.

The procedure for isolation of sapogenins from alfalfa roots flour was essentially the same as described above, except that the step of precipitation with cholesterol was omitted. The sapogenin mixtures thus obtained were then subjected to the quantitative determination of medicagenic acid as described above.

Tlc of Saponins and Sapogenins. This was performed as described by Shany *et al.* (1970b) and Gestetner *et al.* (1971a).

RESULTS AND DISCUSSION

In order to ascertain that except for medicagenic acid the saponin extracts did not contain other titrable substances, the extracts were subjected to tlc, from which the different regions were eluted and titrated separately, as well as after being combined. In the case of the root extract, it was found that only the region corresponding to medicagenic acid was acid. A similar experiment with an extract from alfalfa tops showed the presence of considerable amounts of interfering material. In order to overcome this obstacle, a step of purification *via* cholesterol precipitation of medicagenic acid containing saponins was introduced. When the saponin thus obtained was subjected to tlc and subsequent elution of the different regions, only the region of medicagenic acid showed acidity.

The titration curve of medicagenic acid shows (Figure 1) two inflection points corresponding to the carboxyl groups and an end point at about 630 mV. The end point was also determined visually with thymolphthalein as indicator. Calculations of carboxyl groups titrated gave similar recoveries of 74.3 and 75.8%, respectively. The water and mineral contents of the isolated medicagenic acid which were determined account for the remaining 25%. The potentiometric titration is essential only when the color of the extract obscures the color transition of the indicator.

The validity of the titrimetric determination of medicagenic acid was checked with samples of medicagenic acid which had been submitted to the treatment employed for saponins, *i.e.*, acid hydrolysis, ether extraction, etc., both in the presence and absence of saponins. These resulted in a nearly complete recovery (95.0–99.7%) of medicagenic acid.

The amount of medicagenic acid in different parts of the alfalfa plant is given in Table I. Since the amount of sugars in medicagenic acid containing alfalfa saponins is known (Gestetner *et al.*, 1971a), the amount of toxic alfalfa saponins can be derived as well. These data are in good agreement with those of Walter *et al.* (1954) and of Morris *et al.* (1961), who estimated the amount of medicagenic acid containing saponins in alfalfa tops and roots gravimetrically.

The data in Table I show that the concentration of medicagenic acid containing saponins is 8–9 times higher in alfalfa roots than in alfalfa tops, thus explaining the greater potency of alfalfa root saponins in hemolysis, in inhibition of larval and fungal development, as well as in the inhibition of cottonseed germination (Gestetner *et al.*, 1971b; Marchaim *et al.*, 1970, 1972; Shany *et al.*, 1970a).

As a great number of triterpene saponins contain sapogenins

bearing carboxyl groups (Steiner and Holtzem, 1955), it is suggested that the titrimetric method described could be used for the determination of saponins from other sources as well.

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PRO-XAN Process: Air Drying of Alfalfa Leaf Protein Concentrate

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Several methods of drying alfalfa leaf protein concentrate (LPC) for use as a pigmenter in poultry rations were investigated. Both a blend-back and a single pass feed system were developed for use with a pilot model of a commercially available rotary air drier. The dark green granular products retain most of the carotenes and xanthophylls originally present and are suitable for use as feed ingredients.

Although a number of methods for the preparation of leaf protein concentrate (LPC) have been described (Cowlishaw *et al.*, 1956; Morrison and Pirie, 1961), it has proven difficult to dry the resultant product economically and still preserve its nutrient content. Because of the need for minimum product alteration and convenience for overseas shipment, much of the experimental product prepared at Rothamsted has been freeze-dried (Pirie, 1969).

Subba Rau and Singh (1970) have observed no differences in the nutritional quality of LPC dried by a number of different methods, including air drying. Air drying is more economical (Arkcoll, 1969) but produces a hard granular material (Morrison and Pirie, 1961). Arkcoll (1969), in laboratory experiments, reported that LPC in the form of a fine powder suitable for food use can be produced by air drying in two steps, provided that a grinding step is inserted between them.

The PRO-XAN process was developed at this laboratory as a feasible commercial method for the production of alfalfa LPC for use in animal feeds (Booth *et al.*, 1972; Halloran, 1972; Kuzmicky *et al.*, 1972). Since LPC is high in xanthophyll and low in fiber, it is useful as a pigmenter in high energy poultry rations (Halloran *et al.*, 1971; Kohler *et al.*, 1968). Previous papers have described the crushing and expression of juice from fresh alfalfa (Knuckles *et al.*, 1970, 1972), the heat coagulation of the protein xanthophyll complex (Spencer *et al.*, 1970, 1971), and the separation of the coagulum from the alfalfa solubles (Lazar *et al.*, 1971). This paper compares several methods for drying LPC and evaluates the effects of drying on the carotenoid content of the products. It should be pointed out that an important difference between the products used in our work and some of the work cited in the literature is that our product contains substantial amounts of soluble solids. LPC designed for edible use is ordinarily washed to reduce the soluble solids content to 1% or less.

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Preparation of Coagulum. Alfalfa juice, prepared as described earlier (Knuckles *et al.*, 1970), was processed within 3 hr after rolling. Coagulation with steam followed the earlier process (Spencer *et al.*, 1971) except that no air was introduced. Wet coagulum collected immediately after separation from the brown juice contains about 85% water. Drained coagulum, containing about 80% water, was collected from the draining reel (Lazar *et al.*, 1971). Dejuiced coagulum was obtained by mechanical expression as described later, and contains about 55% water.

Freeze Drying. Wet coagulum was frozen in thin layers and dried in a RePP model 15 FFD sublimator at a shelf temperature of +75°F.

Spray Drying. A gas fired, cocurrent flow spray drier (Bowen Engineering Co.) was used. The outer conical shell of the drier was 2.5 ft at maximum diameter by 5.5 ft high. Prior to drying, the coagulum was put through a Waring Blendor and a Charlotte colloid mill to produce a smooth creamy liquid. The liquid was introduced into the drier through a two-fluid nozzle by a positive displacement gear pump at a rate of 120 ml/min. Air at 65 psig was used at the nozzle for atomization. The drier was operated at an inlet temperature of 450°F, and an outlet temperature of 210–240°F.

Drum Drying. A steam-heated Buflovak pilot plant model