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PLANT ANALYSIS

Determination of Saponins in Alfalfa

G. R. VAN ATTA, JACK GUGGOLZ, and C. RAY THOMPSON[1]

Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany 10, Calif.

A reasonably rapid procedure is presented for determining total saponins in alfalfa. Selective influences of several solvent systems upon the adsorptive behavior of activated carbon are used to separate saponins from nonsaponins. Applied to plant samples the method yields reproducible results, although the analytical values are somewhat higher than the true values. The method has been used in studying the relation between chick growth and saponin content of alfalfa in feeds.

UNDER SOME CIRCUMSTANCES triterpenoid saponins of alfalfa (*5, 7*) can inhibit growth in chicks and reduce egg production by hens (*1, 3*). There is also evidence that alfalfa saponins and those of some other legume forages may contribute to the occurrence of bloat in ruminants (*2, 6*).

Investigations into the importance of saponins in dehydrated alfalfa fed to chicks created a need for a quantitative method of determining alfalfa saponins.

Because alfalfa contains a number of different saponins in unknown and presumably varying proportions (*6*) as well as other substances of similar properties, the early methods of assay which depend upon hemolytic properties or surface activities of saponins (*4*) are not applicable. The tendency of cholesterol to form addition compounds with saponins has been used to recover saponins from alfalfa (*6, 7*), but attempts to base an analytical method on the same principle were abandoned when preferential recovery of some and incomplete recovery of all alfalfa saponins could not be avoided. For a time a method was used whereby sapogenins rather than intact saponins were estimated (*6*). However, the procedure required much precise manipulation and far too much time for routine application.

The method presented here uses selective influences of several solvent systems upon the adsorptive behavior of activated carbon toward saponins and other alfalfa extractives. It allows estimation of saponins rather than sapogenins with fair rapidity and with sufficient accuracy for use in studies of their occurrence in alfalfa and their significance in animal diets.

[1] Present address, University of California, Riverside, Calif.

Procedure

Analytical Method. In a 1-liter Erlenmeyer flask, mix 15 grams of dried, coarsely ground or chopped sample with 75 ml. of water. Let the mixture stand 5 hours. Add 210 ml. of 95% ethyl alcohol, mix by swirling, stopper, and let the mixture stand 20 hours; then add and mix 53 ml. of 95% ethyl alcohol and 162 ml. of water. The volume of liquid added is now 500 ml. and the concentration of ethyl alcohol is 50%. Allow 1 hour for equilibration, then suction-filter the liquid through medium speed paper. If the dry plant material is finely ground, as little as 3 grams of sample and proportionally smaller volumes of liquids may be used to prepare this extract solution.

To a 50-ml. portion of the alcoholic extract solution (equivalent to 1.5 grams of sample) add 1.0 gram of activated carbon and warm over steam with occasional stirring for 15 minutes. Suction-filter through a 5.5-cm. medium speed filter paper. Complete the transfer and wash with 100 ml. of 50% ethyl alcohol. Evaporate the filtrate and washings to near dryness over steam, warm the residue with 20 ml. of water until solution is complete, and then add 1.5 grams of activated carbon. Stir the mixture occasionally and continue warming over steam for 5 minutes. Suction-filter through a 5.5-cm. smooth, hardened paper. If the filtrate contains carbon, return it to the funnel, repeating the filtration if necessary until a carbon-free filtrate is obtained. Wash the carbon in the funnel with four 20-ml. portions of water followed by two 20-ml. portions each of 10 and 20% ethyl alcohol. Discard the filtrate and washings. To elute adsorbed saponins from the carbon wash the filter with 200 ml. of a mixture of pyridine (purified grade) and absolute ethyl alcohol, 3 to 7 (v./v.). Throughout filtration and elution do not permit the liquid level to reach the filter cake between additions. Evaporate the pyridine-alcohol eluate in a tared dish, vacuum-dry the residue 16 hours at 65° C., and weigh as saponins.

Commercial activated carbons differ in their suitability for this procedure not only by brands and grades, but also within the same brand and grade. Each lot that is to be used has to be selected by test. Mixed alfalfa saponins for testing activated carbons can be prepared according to the procedure that follows.

Preparative Recovery of Saponins

Heat a continuously stirred mixture of 500 grams of alfalfa meal and 4 liters of water to 95° C. and suction-filter the hot liquid. Heat the filtrate to boiling and add 80 grams of powdered cholesterol. Stir and boil the mixture gently for 5 minutes, then cool it to room temperature and add 40 grams of analytical grade diatomaceous earth. In a 15-cm. Büchner funnel, suction-filter the liquid through medium speed paper that has been precoated with 20 grams of analytical grade diatomaceous earth in the form of a filter cake. To avoid

blinding the filter it is important to use only moderate suction and to pour the thoroughly stirred suspension onto the filter in very small portions, allowing each liquid portion to pass into the filter cake before adding more. Wash the filter cake with small portions of water until the washings are colorless. About 150 ml. of water will be required. Avoid excessive washing, which will result in loss of saponins. Transfer the drained filter cake to a shallow container and dry it in an oven at about 75° C. Extract most of the cholesterol from the dried cake by mixing it with at least three successive 500-ml. portions of U.S.P. grade diethyl ether, suction-filtering each mixture separately and washing each filter cake in the funnel with 50 to 100 ml. of ether. Remove ether from the last filter cake by drawing air through it. Remove the last of the cholesterol from the filter cake by extracting with chloroform for 24 hours in Soxhlet-type extractors and dry the extracted cake.

Recover saponins from the extracted filter cake by mixing the cake with 350 ml. of hot water followed by suction filtration. Wash the drained cake with three 100-ml. portions of hot water. Add to the filtrate 1.1 times its own volume of 95% ethyl alcohol and 2.0 grams of activated carbon, warm the mixture over steam for 10 minutes, stirring occasionally, and suction-filter through retentive paper. Evaporate the filtrate and vacuum-dry the residue at 65° C. For convenience saponins obtained in this way will be referred to as "cholesteride saponins."

Yields of cholesteride saponins vary with alfalfa meals and details of preparation. They are always less than the total saponins in the respective meals. Thus, in a particular example, 0.4% of cholesteride saponins was obtained from a meal that contained about 1.5% of saponins.

Testing Activated Carbon

Warm about 500 mg. of cholesteride saponins in water and dilute to 200 ml. in a volumetric flask. To determine the nonvolatile matter in this stock saponin solution evaporate a 20-ml. portion and weigh the residue after vacuum drying 16 hours at 65° C.

For each carbon test mix 20 ml. of the stock solution with 1.5 grams of carbon, heat the mixture, filter, and elute as in the analytical procedure. Combine and evaporate the initial filtrate, water washings, and the water–ethyl alcohol eluates. Vacuum-dry and weigh the residue which will be comprised in part of soluble impurities in the cholesteride saponins and in part of soluble materials leached from the carbon. If the carbon is satisfactory this residue will not contain saponin. Test the dried residue qualitatively for saponins by adding to it several drops of acetic anhydride followed by several drops of concentrated sulfuric acid. Appearance of a red color after a few seconds indicates the presence of saponin.

A blank analysis starting with 20 ml. of water instead of stock solution will determine the weights of nonvolatile materials derived from the solvents and the carbon. These weights should be applied as corrections to the results of the analysis of the stock solution. If the carbon being tested is satisfactory, the weight of nonvolatile material found in 20 ml. of stock solution minus the weight of the soluble nonsaponin impurities it contained will match the corrected weight of the evaporation residue from the pyridine–ethyl alcohol eluate.

Carbons that are initially unsatisfactory because they release saponins in the water–ethyl alcohol eluates can sometimes be made suitable for use by first washing them with acid and then reactivating them with heat. The carbon in 1*N* hydrochloric acid is held at 80° C. for 1/2 hour, suction-filtered, washed with distilled water until the washings are neutral, and then kept in an oven at 100° C. for 15 hours.

Attempts to adjust the adsorbant properties of carbons that are initially too retentive—i.e., those carbons that do not release saponins completely in the ethyl alcohol–pyridine eluate—have not been successful.

Results and Discussion

Choice of Carbon. Despite numerous trials, no eluent systems were discovered that would obviate selection of activated carbons by actual tests, nor was any other criterion found that would assist in the selection. The degrees to which differing carbon qualities may influence completeness of saponin recovery are illustrated in Table I. The table includes results obtained with two lots of carbon before and after they were acid-washed and reactivated. Pretreatment was effective with one of these carbons, but not with the other.

Accuracy and Reproducibility of Results. Saponin contents of alfalfa samples found by the present method are somewhat higher than the true values. A method employing streak chromatograms on paper whereby the purity of the analytical products and hence the true saponin contents of the original samples can be determined is currently being developed and will be reported at a later date. A tentative estimate reached by the later technique places the purity of the saponins obtained by the present procedure at about 75%. However, purity of the analytical products does not vary greatly between various samples.

Employing the method described here an operator analyzed 110 alfalfa samples, of which 16 were assayed in duplicate and one in triplicate in 23 working days. In addition the same analyst determined the moisture contents of all but 19 of the samples in the same period. Results obtained by analyzing 16 alfalfa samples in duplicate and one sample in triplicate displayed a standard deviation, s, of 0.11% and a confidence limit of 0.23% at the 5% level. The percentages of saponins found in this series ranged from 1.39 to 3.49%.

Degrees of agreement between separate determinations were also tested by an experiment in which two alfalfa meals of differing saponin contents and three

Table I. Recovery of Adsorbed Saponins from Different Lots of Activated Carbon

Stock Solution	Carbon Lot	Saponins, Mg. Test portion	Saponins, Mg. Recovered[a]
1	None	46.2	
	A		46.1
	B		39.5
	C		38.7
	D		44.7
	E		35.4[b]
	E[c]		39.2
	F		30.1[b]
	F[c]		45.9
2	None	34.4	
	G		33.9

[a] Corrected for reagent and carbon blanks.
[b] Qualitative test showed some saponin was lost in 20% ethyl alcohol eluate.
[c] Acid-washed and reactivated.

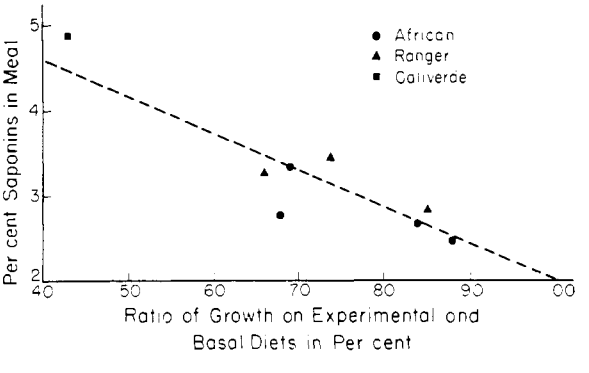

Figure 1. Comparison of chick growth with alfalfas of different saponin contents

mixtures of the same meals were analyzed. The weights of saponins recovered from the mixtures were almost perfectly proportional to the percentages of the two meals in the samples.

Application of Saponin Determinations to Study of Chick-Growth Inhibition. An example of the utilization of saponin determinations in studying the influence of alfalfa saponins in diets upon growth of chicks is shown in Figure 1. In the figure, relative growth in 6 weeks is plotted against percentages of saponins in alfalfas fed at 20% levels in experimental diets. Each point on the graph represents the average for a group of 20 chicks.

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NUTRIENTS IN SEED MEALS

Amino Acid Composition of Twenty-Seven Selected Seed Meals

C. H. VanETTEN, R. W. MILLER, and I. A. WOLFF

Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Ill.

QUENTIN JONES

Crops Research Service, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.

Seed meals obtained from 27 genera of 13 botanical families were analyzed for their acid-stable amino acid content by the Moore, Spackman, and Stein ion exchange chromatographic method and for tryptophan by a modified Spies and Chambers procedure. Comparison of the amino acid compositions with the requirements for optimum growth of the rat and the chick showed some of the seed meals to be well balanced. The majority were deficient in methionine-cystine, lysine, or both. Lysine content varied from 2.5 to 6.9 grams and methionine from 0.5 to 2.3 grams per 16 grams of nitrogen. Three genera from the legume family contained canavanine, which was identified after isolation from *Sesbania exaltata*.

New crops research in the Agricultural Research Service, U. S. Department of Agriculture, has a major objective of discovering plants that may be advantageously grown by the farmer in place of commodities like corn and wheat now in surplus. One large part of this program consists of determining the chemical composition of a wide variety of plants. Special emphasis is placed on quantitative analysis for compounds of known practical significance, such as amino acids. Also important is the discovery of unknown compounds that may have economic potential, and industrial uses appropriate to the properties found or developed.

Ion exchange chromatographic methods of quantitative analysis for amino acids and related compounds are ideal for determining the amino acid composition of plant material. In addition, these methods detect unknown nitrogen-containing compounds which form a color with ninhydrin during analysis. This report contains analytical results from the determination of the amino acid composition of seeds from 27 selected plant species.

Materials and Methods

Seed Selection and Preparation. Factors considered in the selection of seed meals from different plant species for analysis were: high protein content; chemical composition, such as high or unique seed oil content; relatively high lysine or methionine, as determined by microbiological assay; lack of amino acid data in the literature for seed from the species reported; and favorable agronomic possibilities of the plant for the temperate zone. One sample from each species was analyzed. Amino acid composition might vary between varieties or with environmental conditions, but such variability is probably of a lesser magnitude than that to be found between species.

Mature, dry seeds were selected for analysis. Easily removable outer seed coverings (fruit or seed coat) were separated, using conventional seed-cleaning equipment. The sample was ground to pass a standard U. S. 40-mesh screen and oil was extracted with petroleum ether (boiling point 33° to 57° C.). The extracted meal was allowed to come to equilibrium with the moisture in ambient air, then sampled for total nitrogen determination and for hydrolysis.

Acid Hydrolysis. The seed meals were hydrolyzed for 24 hours under reflux with constant boiling hydrochloric acid, redistilled in glass. Excess acid, 250 ml. per 0.5 ± 0.2 gram of meal as recommended by Dustin *et al.* (2), prevented excessive humin formation in most cases. The acid was removed under vacuum to near dryness with a rotary evaporator, followed by evaporation of three small volumes of added water. The humin was removed by centrifugation; its nitrogen content was determined by the Kjeldahl method on the air-dried material collected in a weighed centrifuge tube. The clear supernatant was made to 50-ml. volume and held in a frozen state if not analyzed within 2 weeks.