hibiting ability depends on the concentration of protein substrate relative to the concentration of carrageenin—and that the degree and character of this inhibition depends on the particular substrate used in the assay for enzymatic activity. The data support the conclusion of Piper and Fenton (*5*) that carrageenin does not react with the active center of the enzyme but inhibits by combining with the substrate. The carrageenin in food products should not interfere with normal peptic digestion of protein, since the concentration of protein is higher and the concentration of carrageenin much lower in such products than in the experimental mixtures where inhibition was demonstrated. Although the exact concentration of protein necessary to prevent inhibition of pepsin by a given concentration of carrageenin depends on the kind of proteins in the mixture, a low concentration (0.25%) of any dietary protein would probably be sufficient at the carrageenin levels used in food products. The conclusions drawn from the experiments in vitro are supported by the data from animal feeding experiments summarized in Table I.

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PLANT ANALYSIS

Supplementary Chromatographic Method for Determining Saponins in Alfalfa

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A supplementary procedure is described for improving accuracy in the gravimetric determination of saponins through application of quantitative paper chromatography.

An earlier gravimetric method for approximate determination of saponins in alfalfa involved adsorption of saponins on carbon and their subsequent recovery by elution (*2*). Complete recovery of saponins was demonstrated; however, results were known to be somewhat higher than the true values. Despite this shortcoming, results obtained by the method could be used in studies of the significance of alfalfa saponins in chick diets. However, the desirability of a more accurate total saponin assay was recognized, and a supplementary chromatographic procedure has been developed for application to the analytical products eluted from carbon. By this supplementary treatment, inaccuracies due to incomplete separation of saponins and nonsaponins can be considerably reduced. Some of the techniques applied in these instances to saponins might be useful in estimating some other minor constituents of plants which present similar obstacles to analysis.

For brevity, the impure saponins eluted from carbon with ethyl alcohol and pyridine in the preceding procedure (*2*) are referred to here as CAP saponins.

Apparatus

Two chromatographic accessories were used, a streaking pipet (Figure 1), and a sample applicator. The sample applicator supports, illuminates, and dries chromatographic paper during sample application. Its novel feature is a traveling clamp that carries the pipet during streaking and automatically lowers and raises it at the ends of sample streaks. Both accessories were manufactured by Research Specialties Co., 200 Garrard Blvd., Richmond, Calif.

Procedure

CAP saponins, while still in the dish in which they had been weighed, were dissolved in 1 to 1.5 ml. of 60% ethyl alcohol. With the sample applicator and streaking pipet, this solution was applied to a sheet of Whatman 3MM paper in a streak parallel to and 2.5 cm. from an edge of the paper. Streak length was limited either by the weight of CAP saponins available or by paper and equipment dimensions. Maximum streak length was about 40 cm. The most suitable density of loading was about 1 mg. of CAP saponins per cm. of streak. To achieve satisfactory loading, it was necessary to draw the pipet along the streak repeatedly with intervals for drying between applications. Uniform loading throughout the streak and absence of sample spreading at the ends of the streak were necessary conditions. Saponin solutions have a strong tendency to form drops at the ends of pipet tips. To prevent this, with consequent spreading at the ends of the streaks, it was necessary to shrink the bore of the pipet tip to not more than 0.13 mm. and to limit its length to not more than 8 mm. The ends of the tips were squared and chamfered around the edges by grinding. Fire polishing instead of grinding was unsatisfactory.

When streaking was completed, the pipet was rinsed with 60% ethyl alcohol. Rinsings were delivered into the dish that initially contained the CAP saponins, the liquid was evaporated, and the dish and residue were vacuum dried at 65° C. and weighed to determine by difference the weight of CAP saponins applied to the paper.

After the length of the streak had been measured and recorded, the width of the paper was accurately trimmed to the ends of the streak, and an ascending chromatogram was developed on the paper. The developing solution used was the upper phase of a *n*-butyl alcohol–1*M* ammonium hydroxide–95% ethyl alcohol (60:30.5:13) solvent mixture. Development was stopped when the liquid front had advanced 15 cm. above the level of the sample streak. Development time at 30° C. was about 3 hours.

Saponins were located on the dried chromatogram by staining a 1-cm. wide test strip that was cut from the top to the bottom of the chromatogram at its middle. The strip was stained by drawing it through a mixture of sulfuric acid and acetic anhydride (*1*). Positions of saponins from alfalfa leaves and stems became visible on the test strip by the appearance of a blue band surmounted by a red- to plum-colored band in the region from about R_f 0.15 to about 0.55. The upper and lower limits of the

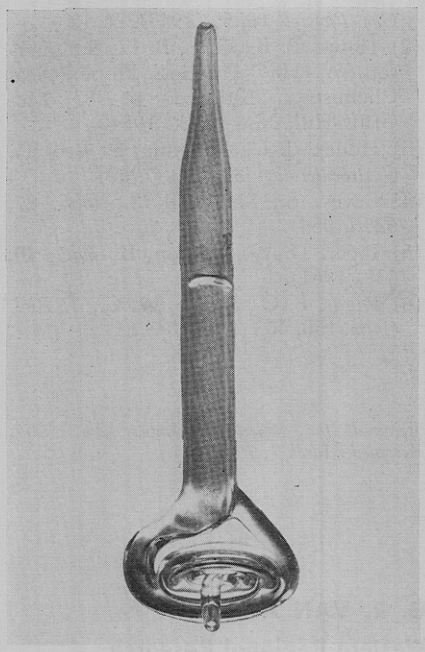

Figure 1. Streaking pipet; over-all length 9 cm.

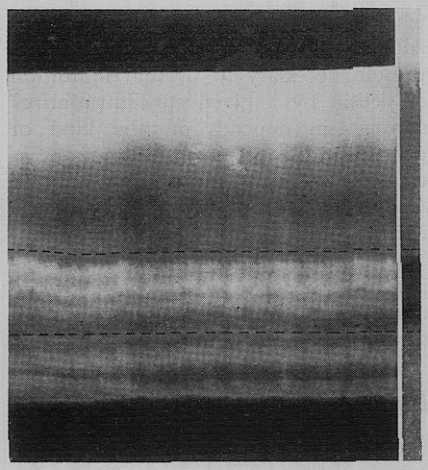

Figure 2. Composite photograph of fluorescent bands on one unstained portion of chromatogram (left) and saponin zone on stained test strip (right)

(Dashed lines mark boundaries of portion taken for recovery of saponins)

saponin zone were then marked in pencil on the two parts of the chromatogram that had not been stained, along the edges of the paper that had adjoined the test strip. When illuminated by a low pressure mercury lamp fitted with a filter that excluded visible light and transmitted in the 2537-A. region, these pieces of the chromatogram displayed a number of horizontal fluorescent bands which, in most instances, were probably produced by phenolic substances. By using appropriate fluorescent bands as guides, the marginal marks at the upper and lower limits of the saponin zone were extended across both sheets. These relationships are illustrated in Figure 2. The area of the saponin zone was excised and cut into small pieces. Saponins were recovered from the pieces by warming them gently for 5 to 7 minutes with each of three successive 40-ml. portions of 60% ethyl alcohol. The extract solutions, recovered by draining, were combined, filtered, and evaporated. The evaporation residue was vacuum dried at 65° C. and weighed. A correction for soluble substances extracted from the paper itself was deducted from the weight of the residue. The size of the correction was determined from the weight of residue obtained when a blank paper was developed, sectioned, and extracted in the same way as the chromatogram. Under our conditions, the correction amounted to 0.0215 mg. per sq. cm. of paper. Usually, the total correction was in the range of 3 to 4 mg. Prewashing the paper to reduce this correction was impractical because, when dried after washing, the papers were not flat enough for uniform streaking.

The weight of CAP saponins applied to the part of the chromatogram taken for saponin recovery was calculated from the total weight of sample applied, the total length of the sample streak and the width of the test strip. The purity of the CAP saponins—i.e., the approximate per cent of saponins in the sample—was calculated from the weight of material recovered from the chromatogram, corrected for the paper blank, and from the sample weight it represented.

Discussion and Results

Estimates of saponin percentages obtained in the manner described here are not completely free from inaccuracy. Evidence for this is the slight color of the final products caused presumably by fluorescent nonsaponins co-existent with saponins in the same zone on the chromatogram. Efforts to eliminate this source of inaccuracy were not successful. Thus, further liquid travel in development of the chromatogram to achieve better separation resulted in diffuse, poorly defined saponin bands. Rechromatography for the same purpose greatly complicates the procedure and introduces additional opportunities for experimental error. The magnitude of the error introduced by the presence of fluorescent materials in the saponin fraction recovered from the paper is not known with certainty, but other chromatographic work at this laboratory with fluorescent materials in alfalfa strongly suggests that it cannot be significant.

Table I. Per Cent Purity of Total Saponins Obtained as Analytical Products by Elution from Carbon with Ethyl Alcohol–Pyridene Solution (*1*)

Alfalfa Variety	Samples	Av. Purity, %	Std. Dev.
Buffalo	12	78.0	4.5
Vernal	15	76.5	5.8
Lahontan	14	68.9	5.8
Caliverde	6	77.7	4.1
DuPuits	6	68.6	5.7
Av.		73.9	5.2

To find out if changing the quantities and proportions of saponins in the starting materials would cause discrepancies in the analytical results, two specimens of alfalfa saponins were used. One of these, a sample of CAP saponins (*A*), had been found by the present method to have a purity of 75.8%. The other specimen (*B*), was recovered by the preparative method described in a previous article (*1*). Its purity was 92.7%. A chromatogram was developed on a paper to which *A* and *B* had been separately applied in a single streak. Loadings on the part of the chromatogram taken for saponin recovery were 32.7 mg. of *A* and 15.1 mg. of *B*. From these weights and the purities of the specimens, the calculated weight of saponins to be recovered was 24.8 mg. plus 14.0 mg., or 38.8 mg. The weight actually recovered was 37.2 mg. The difference is regarded as within the expected limits of experimental error.

The purity of CAP saponins obtained in analyses of a series of 53 samples of alfalfa was examined by the present method with results as summarized in Table I. Individual CAP saponins contents of the 53 samples ranged from 1.74% to 3.92%.

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