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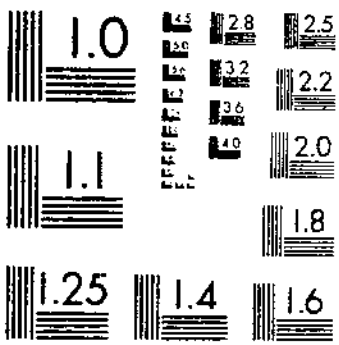
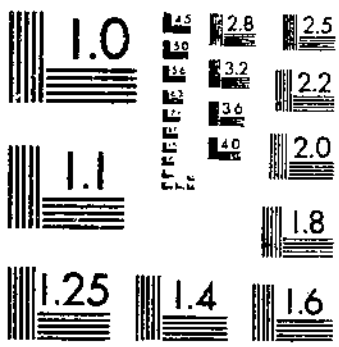


MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A



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VARIATION IN COUMESTROL CONTENT OF ALFALFA

As Related to Location, Variety, Cutting, Year, Stage of Growth, and Disease

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Variation in Coumestrol Content of Alfalfa as Related to Location, Variety, Cutting, Year, Stage of Growth, and Disease

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NATURALLY OCCURRING ESTROGENS IN FEED

Estrogenic activity has been noted in more than 50 species in 25 families of plants (*13*).[6] It was reported in roots, tubers, stems, leaves, flowers, and seeds. However, since in most cases, relatively low potency was found, little significance was attached to it, even though some of the plants provide common foodstuffs.

Although estrogens had been reported in forage crops, interest in them did not develop until a severe outbreak of sterility in ewes in a large area of Australia was attributed to subterranean clover (*Trifolium subterraneum* L.) with estrogenic activity (*7*). Australian workers showed that subterranean clover contained large amounts of genistein (*14*), an isoflavone with estrogenic activity (*12*). Subterranean clover was also found to contain the isoflavones formononetin (*36*), biochanin A (*36*), and daidzein (*20*). Most recent information indicates that formononetin is the principal estrogen in that legume.[7]

Since alfalfa (*Medicago sativa* L.) and Ladino clover (*Trifolium repens* L.) were more economically important than subterranean clover in the United States, workers at the Western Regional Research Laboratory of the U.S. Department of Agriculture undertook investigations of estrogenic compounds in these two forages. In addition to the isoflavone estrogens, a new and more potent estrogen was isolated in pure form (*9, 10*) and characterized (*11*).

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[5] Credit is due B. L. Norwood, Crops Research Division, and E. J. Koch, Biometrical Services, ARS, Beltsville, Md., for assistance on data processing and analyses; D. White, Western Utilization Research and Development Division, ARS, Albany, Calif., for laboratory assistance; F. I. Frosheiser and J. H. Graham, Crops Research Division, ARS, St. Paul, Minn., and University Park, Pa., respectively, for providing alfalfa samples differentiating damage from foliar diseases; A. W. Hovin, University Park, Pa., for assistance with field experiments; and American Dehydrators Association for providing some of the funds for coumestrol analysis.

[6] Italic numbers in parentheses refer to Literature Cited, p. 43.

[7] Letter communication from David Bennett, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia.

Because of its coumarinlike structure, the compound was named coumestrol. It was later shown to be about 30 to 40 times more potent in mouse uterine-weight assays than isoflavone estrogens. Coumestrol content of potent dehydrated alfalfa samples accounted for 90 percent or more of the estrogenic activity. The four isoflavones mentioned contributed to, but did not completely account for, the remaining activity (*20*).

A sensitive paper chromatographic assay procedure based on the intense blue fluorescence of coumestrol was developed (*26*). It permitted rapid assay of the many samples described in this bulletin. Prior to development of this method, some studies had been conducted with mouse assay procedures. Earlier work showed that estrogenic activity was extremely variable in different alfalfa samples. Also, more mature alfalfa tended to have higher estrogenic potencies than younger forage (*3, 8, 24, 25, 35*).

In this study, interest in learning more of the causes for variability in alfalfa estrogen content was based on two developments. First, breeding difficulties had been reported in livestock grazing on alfalfa and other legume forage crops grown commonly in the United States (*1, 7, 16, 18, 48*). These observations indicated a need to avoid high-estrogen alfalfa with breeding stock. Second, many feed-lot steers were being fed the synthetic estrogen diethylstilbestrol to improve growth rate and efficiency of feed conversion. This suggested that processed forage products rich in estrogen might find a ready market in rations for fattening steers, wethers, and perhaps poultry.

Preliminary tests with high-estrogen forages or extracts prepared from them indicated that plant estrogens had growth-promoting effects on sheep (*33, 34, 44*). Results with beef cattle over a period of several years suggested that high-estrogen alfalfa stimulated growth of steers as did stilbestrol (*29, 30*). However, in later experiments the same workers were unable to repeat their results (*28*). Further work is needed to establish the activity and potential utility of plant estrogens as growth stimulants for ruminants, especially cattle.

STAGE OF HARVESTING ALFALFA AS RELATED TO YIELD, QUALITY, AND PERSISTENCE

Since some work has indicated that estrogenic potency of forage varies with maturity, the feasibility of altering coumestrol content of alfalfa by shifting the stage of harvesting is of interest. Previous work has shown that yield, digestibility, chemical composition, and stand persistence are principal considerations for determining the best time to cut alfalfa. Literature on this subject is extensive and is reviewed only briefly.

Dry-matter yields consistently increased with maturity (*6, 19, 22, 31, 32, 37, 38, 39, 40, 41, 45, 47*). Relative yields of hay cut at the bud, 1/10-bloom, and full-bloom stages were, respectively, 100, 121, and 129 percent in Kansas (*37*) and 100, 116, and 123 percent in Oklahoma (*19*). In Nebraska (*22*) relative yields of hay cut at the prebloom, 1/10-bloom, and new-growth stages were 100, 112, and 117 percent, respectively.

Although alfalfa yields increased with advancing maturity, percentage of digestible energy decreased. Estimated digestible dry matter declined with advance in maturity in artificial rumen digestion

tests (*6*). In feeding tests, digestible nutrients also decreased as maturity increased (*15, 31, 37*). Relative yields of digestible nutrients per acre from hay cut at initial-bloom, half-bloom, and full-bloom stages were 100, 95, and 70 percent, respectively, in Montana (*15*). In a California study (*31*) relative production of total digestible nutrients per acre was 100, 113, and 125 percent, respectively, for hay cut in the prebud, bud, and $\frac{1}{10}$-bloom stages.

Steer-feeding tests in Kansas (*37*) showed that feed value of alfalfa hay decreased materially with delay in cutting. Dairy cattle-feeding tests in Montana (*15*) showed that milk and butterfat production declined as alfalfa hay maturity increased. California experiments (*31*) with sheep and cattle demonstrated that feed quality decreased as hay maturity increased; however, swine did not respond to differences in hay maturity.

Percent protein also decreased as alfalfa forage increased in maturity (*15, 19, 22, 31, 32, 37, 38, 39, 41, 47*). Relative yields of protein from hay cut at the prebloom, initial-bloom, $\frac{1}{10}$-bloom, half-bloom, full-bloom, and new-growth stages were 100, 93, 98, 97, 87, and 99 percent, respectively, in Nebraska (*22*). In Montana (*15*) relative yields of protein from hay cut at the initial-bloom, half-bloom, and full-bloom stages were 100, 97, and 68 percent, respectively. In Kansas (*37*) relative yields of protein from hay cut at the bud, $\frac{1}{10}$-bloom, and full-bloom stages were 100, 115, and 113 percent, respectively. Wisconsin results (*38, 39, 41*) showed that higher yields of protein per acre were generally obtained from three or four cuttings per year than two. Protein yields per acre varied with the date of cutting and the variety. Yields per acre in percentage ranged from 88 for two-cut to 146 for three-cut to 158 for four-cut schedules (*39*).

Crude fiber content increased with maturity (*6, 15, 19, 22, 32, 37, 38, 47*) and was higher in the first than last cutting in Kansas (*37*) and Montana (*15*), where proportion of stems had a direct bearing on fiber content. Crude fiber content was highly and inversely correlated with palatability of hay (*31*).

In Nebraska (*22*) fat content of forage was not affected by maturity. In Wisconsin early and late June cuttings did not differ in percentage of fat (*38*). Although percentage of fat declined with maturity in the spring, there was no pronounced trend in the summer. Highest amount of fat per acre was obtained at mature stages of growth (*47*).

Proportion of leaves and stems was often cited as the principal factor in quality. In a Nebraska experiment (*22*) the proportion of leaves in hay harvested at the prebloom, initial-bloom, $\frac{1}{10}$-bloom, half-bloom, seed, and new-growth stages was 57, 56, 53, 49, 33, and 53 percent, respectively. In Oklahoma (*19*) percentage of leaves in hay cut at the prebud, $\frac{1}{10}$-bloom, and $\frac{1}{2}$-bloom stages ranged from 61 to 65, 50 to 61, and 46 to 54, respectively. Similar results were reported from Kansas (*37*). More ash, protein, and nitrogen-free extract of alfalfa hay were found in leaves than in stems. More fiber was in stems than in leaves (*19, 22, 37*). Leafiness and protein contents were positively correlated (r=0.86) (*23*).

Seasonal yields and persistence of stand were reduced when alfalfa was cut too frequently. An extensive review of previous work on this subject is available (*40*). The alfalfa plant utilizes sugars and starch stored in the roots and crown to produce new growth. About the time flower buds are developing, the process reverses and food reserves are

stored. When alfalfa is cut repeatedly in the bud stage, or earlier, plants are weakened and die prematurely. It has been generally concluded that alfalfa should be cut in the 10-percent bloom stage to obtain high yields of good-quality hay without appreciably reducing stand persistence. The number of cuttings obtainable in a season is determined primarily by the length of the growing season and other characteristics of the growing period, such as temperature and moisture.

OBJECTIVES OF STUDY

Although previous reports indicated that plant estrogens might affect reproduction and stimulate growth in farm animals, information was lacking on genetic, physiological, and cultural factors that might affect estrogenic activity of alfalfa. Accordingly, this study on the magnitude and nature of variability of coumestrol content of alfalfa was undertaken. Information sought would be especially valuable in ascertaining whether breeding, physiology, management, or possibly another discipline would be the most profitable area for further research to control or alter coumestrol content. New findings will ultimately help the livestock producer.

Major objectives of this work were to study (1) variation in coumestrol content of five varieties of alfalfa harvested at the 1/10-bloom stage in cuttings in each of 2 years at seven locations, (2) the relationship of growth stage to coumestrol content by harvesting two varieties at six stages of growth at three locations, (3) in a preliminary experiment, the effect of variation in light intensity on coumestrol content, and (4) the effects of foliar pathogens on coumestrol content. The last objective was added after research on the first two objectives, together with information from a study of coumestrol variation in controlled environments (*27*), provided information that implicated micro-organisms.

Alfalfa is the most economically important legume grown for hay in the United States. The 1962 estimate by the U.S. Department of Agriculture (*46*) indicated total alfalfa acreage in the United States was about 29 million, 64 percent in the 12 North Central States, but some in nearly every State. Thus, areas of production differ widely in climates, soils, and other ecological characteristics.

VARIATION IN COUMESTROL CONTENT AT ONE-TENTH-BLOOM STAGE AS AFFECTED BY VARIETY, CUTTING, LOCATION, AND YEAR

Objectives of this part of the study were (1) to characterize variation in coumestrol content of alfalfa harvested at the 1/10-bloom stage from a set of varieties, cuttings, locations, and years, and (2) to ascertain the relationship of coumestrol content to other characters studied.

Materials and Methods

Planting and Harvesting

The varieties "Buffalo," "Du Puits," "Lahontan," "Ranger," and "Vernal" were chosen as representative of the germ plasm found in principal alfalfa varieties grown in the United States. These five

varieties were seeded in replicated plots at Davis, Calif., Ames, Iowa, Manhattan, Kans., Lincoln, Nebr., Raleigh, N.C., University Park, Pa., and Logan, Utah. Plots were harvested in 1960 and 1961. Data from the five varieties, three cuttings each year, seven locations, and 2 years constituted the "core" of the experiment on which combined statistical analyses were conducted. Additional cuttings that were harvested and analyzed for these five varieties, based on four replications, were California two and Kansas and North Carolina one each. Plot size and other details are given in table 1.

TABLE 1.—*Soil types, seeding dates, and other alfalfa field-plot data for 7 locations*

State	Soil type	Date seeded	Method of seeding	Plot size
		1959		*Feet*
California	Yolo loam	Oct. 15	Broadcast	5×20
Iowa	Webster silt loam	Aug. 18	Multiple row, drilled.	5×17
Kansas	Sarpy fine sandy loam	Sept. 3[1]	do	5×20
Nebraska	Sharpsburg silty clay	Aug. 20	Broadcast	5×20
North Carolina	Georgeville silty clay loam.	Sept. 4	do	5×20
Pennsylvania	Hagerstown clay loam	Aug. 4	do	5×20
Utah	Salt Lake silt loam	Sept. 2	Multiple row, drilled.	5×20

[1] 1958.

Immediately prior to harvesting each crop, the following data were recorded:

(1) *Plant height.*—Average height of plants within each plot was recorded in inches.

(2) *Defoliation score.*—Plots were scored from 0 to 7, representing 0- to 70-percent or greater defoliation, respectively.

(3) *Average soil moisture conditions on day of harvest and previous day.*—Very droughty=1, soil moisture not limiting for growth=3, and conditions between 1 and 3=2.

(4) *Amount of sunshine on day of harvest and previous day.*—Sunshine on day of harvest and day previous=1; sunshine on day of harvest, cloudy on previous day=2; cloudy on day of harvest, sunshine on previous day=3; and cloudy on both days=4.

(5) *Maximum air temperature.*—Average for day of harvest and previous day.

(6) *Minimum air temperature.*—Average for two nights previous to harvest.

Forage was harvested at the $\frac{1}{10}$-bloom stage; i.e., when either one-tenth of the plants had at least one stem with one or more open florets or when 25 percent of the plants had crown shoots one-fourth to one-half inch in height. Cutting height was 2 to 3 inches. Harvesting was generally between 10 and 11 a.m., local standard time. At Lincoln, Manhattan, and Davis, varieties were cut on different days to adjust for differences in time of flowering. At the other four locations, all varieties were cut on the same date. This deviation in harvesting procedure among locations did not appear to introduce any variation of consequence.

When the alfalfa was harvested, a representative sample of green forage was cut immediately into stem pieces about 2 inches long. One-quart glass jars containing 600 cc. of alcohol (ethanol in 1960 and methanol in 1961) were tightly filled with chopped alfalfa until the alcohol was within one-half inch of the jar lip and then sealed. Appropriate weights were recorded before and after filling to determine green sample weight. After each cutting, samples were shipped to the Western Regional Research Laboratory at Albany, Calif., for coumestrol determination.

Also, when plots were harvested, a sample of forage weighing about 1,000 grams was obtained from each plot for moisture determination. Each sample was cleaned of weeds or other foreign material and dried immediately at temperatures between 135° and 160° F., except in Nebraska where 190° was used.

In 1960, replications 1 and 2 were composited, as were replications 3 and 4, to give two replications for routine feed analyses. Feed analyses were not made on samples collected in 1961. Statistical computations on feed analyses data were based on two replications, whereas four replications were used in statistical analyses of data for coumestrol, height, and defoliation.

Analytical Procedure for Coumestrol Determination

On arrival at the Albany laboratory, jars were reweighed to determine possible loss from leakage. Samples were analyzed using modifications of the procedure of Livingston et al. (*26*). Under the conditions described above, sufficient time elapsed during storage of the sample in solvent for complete solution of the coumestrol. Hence, an aliquot was taken directly from the mason jar for analysis.

Where ethanol was used, the supernatant from the jar was filtered through an 18.5-cm. filter (E and D 515). One hundred ml. of the filtered extract was shaken with 35 ml. of Skellysolve B [6] in a 250-ml. separatory funnel. The lower layer was drained off into a second separatory funnel and reextracted with a second 35 ml. of Skellysolve B. After separating, a third Skellysolve B extraction was carried out in the same manner. The Skellysolve extracts were discarded. The lower phase was concentrated in a Rinco vacuum rotary evaporator to a volume of about 25 ml. This solution was then extracted in a separatory funnel with three successive 8-ml. parts of ethyl ether. The combined ether extracts were concentrated to about 3 ml. and transferred to a 10-ml. volumetric flask using absolute alcohol for transfer. After diluting to volume with absolute alcohol, the solution was filtered to remove sediment.

The solution was spotted on an origin line drawn on Whatman 1 chromatographic filter paper (40×57 cm.). Two levels of extract (4 and 12 lambda) and three levels of coumestrol standard (2, 4, and 6 lambda of 100 μg. per milliliter of solution) were spotted systematically on the paper, coumestrol at positions 1, 4, and 7 and unknowns randomly between. After drying at room temperature, the paper was developed by ascending chromatography using a mixture of 50 parts

[6] Trade names are used in this publication solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement by the Department over other products not mentioned.

by volume of acetic acid, 35 parts of water, and 15 parts of concentrated hydrochloric acid as developer. When the solvent front had almost reached the top of the paper, the paper was removed from the tank and dried for 24 hours in a chromatographic drying chamber.

Intensity of fluorescence of coumestrol spots was determined with a photofluorometer (*4*), and coumestrol content of unknowns was calculated from a standard curve plotted from the pure coumestrol readings. Results were expressed on a dry-matter basis calculated from moisture determinations made at field stations.

During analysis of the 1960 samples, it was learned that less interfering material was extracted if methanol were used as the extractant instead of ethanol. For that reason, green alfalfa samples were collected in methanol in 1961, and the methanol extract was spotted directly on chromatographic paper. Also, the developing solvent was changed to 50 percent of acetic acid and 50 percent of water. Development and fluorometry readings were carried out as before. These modifications eliminated tedious steps in the washing and concentration procedures.

Procedure for Other Chemical Determinations

In addition to analysis for coumestrol content, samples obtained in 1960 were subjected to a feed analysis, which included determinations for percentages of protein ($N \times 6.25$), crude fiber, ash, fat, and moisture. Nitrogen-free extract was calculated by difference. Methods used for feed analyses were those of the American Association of Cereal Chemists (*2*). Each chemical constituent was expressed in percentage on a moisture-free basis. Commercial facilities were used for chemical analyses other than coumestrol.

Statistical Procedures

Combined Analyses Over Locations and Years.—Data from the "core" of the experiment were subjected to a combined analysis of variance. The expected mean squares for this analysis are shown in table 2.

Appropriate linear functions of mean squares were used to estimate each variance component. A plot component was computed by adding together all components except that for the cuttings main effect. Individual variance components were expressed as a percentage of the plot component. A plot component computed in this manner arises from variation among plots within cuttings.

In analyses of data for individual locations, all cuttings were included, except the fifth cutting at Davis, Calif., in 1960.

Determining Associations Between Characters.—The sum of squares for each main effect and interaction in the "core" analysis, except those involving replications, were subdivided into the part due to linear regression and the part due to deviations from linear regression for study of the relationship between coumestrol and other characters. The mean square for deviations from linear regression for each effect was tested with the same mean square that was used for testing the effect. If the deviation from regression mean square were significant, it was used to test the significance of the regression component; if not, the error term for deviations was used.

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TABLE 2.—*Expected mean squares for analysis of variance of coumestrol (p.p.m.) in alfalfa from 5 varieties and 3 cuttings harvested in each of 2 years at 7 locations* [1]

Source	d.f.	Expected mean square
Locations (L)	6	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 6\sigma^2_{VR(L)} + 12\sigma^2_{VYL} + 24\sigma^2_{VL} + 60\sigma^2_{YL} + 15\sigma^2_{YR(L)} + 30\sigma^2_{R(L)} + 120\sigma^2_L$
Replications in locations (R (L))	21	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 6\sigma^2_{VR(L)} + 15\sigma^2_{YR(L)} + 30\sigma^2_{R(L)}$
Years (Y)	1	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 12\sigma_{VYL} + 84\sigma^2_{VY} + 15\sigma^2_{YR(L)} + 60\sigma^2_{YL} + 420\sigma^2_Y$
Y×L	6	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 15\sigma^2_{YR(L)} + 12\sigma^2_{VYL} + 60\sigma^2_{YL}$
Y×R (L)	21	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 15\sigma^2_{YR(L)}$
Varieties (V)	4	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 6\sigma^2_{VR(L)} + 12\sigma^2_{VYL} + 84\sigma^2_{VY} + 24\sigma^2_{VL} + 168\sigma^2_V$
V×L	24	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 6\sigma^2_{VR(L)} + 12\sigma^2_{VYL} + 24\sigma^2_{VL}$
V×Y	4	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 12\sigma^2_{VYL} + 84\sigma^2_{VY}$
V×Y×L	24	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 12\sigma^2_{VYL}$
V×R (L)	84	$\sigma^2_e + 3\sigma^2_{VYR(L)} + 6\sigma^2_{VR(L)}$
V×Y×R (L)	84	$\sigma^2_e + 3\sigma^2_{VYR(L)}$
Cuttings (C)	2	$\sigma^2_e + 4\sigma^2_{CVYL} + 28\sigma^2_{CVY} + 8\sigma^2_{CVL} + 56\sigma^2_{CV} + 20\sigma^2_{CYL} + 140\sigma^2_{CY} + 40\sigma^2_{CL} + 280\Sigma C^2_i$
C×L	12	$\sigma^2_e + 4\sigma^2_{CVYL} + 8\sigma^2_{CVL} + 20\sigma^2_{CYL} + 40\sigma^2_{CL}$
C×Y	2	$\sigma^2_e + 4\sigma^2_{CVYL} + 28\sigma^2_{CVY} + 20\sigma^2_{CYL} + 140\sigma^2_{CY}$
C×Y×L	12	$\sigma^2_e + 4\sigma^2_{CVYL} + 20\sigma^2_{CYL}$
C×V	8	$\sigma^2_e + 4\sigma^2_{CVYL} + 28\sigma^2_{CVY} + 8\sigma^2_{CVL} + 56\sigma^2_{CV}$
C×V×L	48	$\sigma^2_e + 4\sigma^2_{CVYL} + 8\sigma^2_{CVL}$
C×V×Y	8	$\sigma^2_e + 4\sigma^2_{CVYL} + 28\sigma^2_{CVY}$
C×V×Y×L	48	$\sigma^2_e + 4\sigma^2_{CVYL}$
Error	420	σ^2_e
Total	839	

[1] Cuttings main effect was assumed fixed; all other main effects were assumed to be random variables. Source of variation associated with each σ^2 is indicated by subscripts.

The test of significance of the mean square for regression is also a test of significance of the correlation coefficient and of the percentage reduction in sums of squares due to regression (r^2) for the particular effect, since the variance component associated with regression $\beta^2\Sigma x^2$ is equivalent algebraically to $p^2\Sigma y^2$. Main effects containing significant interactions not included in any one mean square were tested using the procedure outlined by Snedecor (*42, p. 362*).

Regression sums of squares for locations, varieties, and cuttings estimate the degree of linear association between coumestrol and the other characters for the respective sources of variation. The sum of squares due to deviations from linear regression measures the variation that is independent of linear association with coumestrol content, when coumestrol is used as the "x" variable. Whether coumestrol is used as the "x" or "y" variable affects the test of significance, since two variables are seldom measured with the same degree of precision. However, the percentage reduction in the sum of squares due to regression will be the same regardless of whether coumestrol is the "x" or "y" variable.

Regression sums of squares for interaction effects can be interpreted as measuring the extent to which the interaction effect for coumestrol varies with the interaction effect for another character.

The regression sums of squares were also obtained for variety, cutting, year, and interaction sources of variation for individual locations. The five "core" varieties and all cuttings at individual locations (except the fifth cutting at Davis, Calif., in 1960) were included in the analyses of individual locations.

Results

Combined Analysis of Coumestrol Data

Mean Squares.—Mean squares for the following sources of variation for coumestrol content of alfalfa at the $\frac{1}{10}$-bloom stage were highly significant, as shown in table 3: Locations, varieties, years × locations, varieties × years × locations, cuttings × years, cuttings × years × locations, and cuttings × varieties × years × locations. Mean squares for other sources of variation were not significant at either the 5- or 1-percent levels.

TABLE 3.—*Mean squares from combined analysis of variance of coumestrol (p.p.m.) in alfalfa*

Source	d.f.	Mean square [1]
Locations (L)	6	200, 924**
Replications in locations (R (L))	21	431
Years (Y)	1	1, 697
Y×L	6	34, 934**
Y×R (L)	21	596
Varieties (V)	4	8, 352**
V×L	24	1, 630
V×Y	4	616
V×Y×L	24	1, 332**
V×R (L)	84	340
V×Y×R (L)	84	468
Cuttings (C)	2	109, 987
C×L	12	29, 580
C×Y	2	222, 528**
C×Y×L	12	29, 214**
C×V	8	3, 754
C×V×L	48	1, 721
C×V×Y	8	3, 170
C×V×Y×L	48	1, 847**
Error	393	514

[1] **=significant at 1-percent level.

Variance Component Estimates.—Components contributing to plot variation were placed in three general classes based on the magnitude of the estimate. Components for locations, cuttings × years, and cuttings × years × locations were similar and collectively accounted for approximately 72 percent of the plot variation, as given in table 4. The second class consisted of components for years × locations, cuttings × varieties × years × locations, and error, and was responsible for 24.5 percent. The third class consisted of the 13 remaining components, the largest of which accounted for only 1.2 percent. The part attributed to varieties accounted for 0.8 percent. As previously noted, the cutting effect was assumed to be fixed and was not included

in the plot component. Estimate of the cutting component was negative; hence, the component was assumed to be zero. As indicated above, however, interactions with cuttings were large and accounted for about one-half of the plot variation.

Average Coumestrol Content.—Coumestrol content for the five varieties averaged over cuttings and locations is summarized in table 5. Differences between varieties were small but significant. Lahontan had the highest average coumestrol content, Du Puits and Vernal were lowest, and Buffalo and Ranger were intermediate. Rank of varieties was not consistent over locations and years, although Lahontan ranked first and Du Puits ranked last more frequently than did the other varieties (table 7). Although differences among varieties were small in relation to some other sources of variation, forage from Lahontan averaged 35 percent more coumestrol than Du Puits.

Cutting averages were not significantly different when averaged over varieties, locations, and years (table 5). In any 1 year, however, cuttings were probably different, but these differences were not consistent for the 2 years; thus a highly significant C×Y interaction resulted (table 3). In 1960, average coumestrol content over locations was lowest at the first cutting and increased with succeeding cuttings. In 1961, on the other hand, coumestrol content was highest at the first cutting, lowest at the second cutting, and intermediate at the third cutting. However, the inconsistent C×Y interaction at each location resulted in a highly significant C×Y×L interaction (table 3). The latter was a major source of variance (table 4).

TABLE 4.—*Variance components for coumestrol content (p.p.m.) estimated from combined analysis of variance at 7 locations*

Component	Estimate	Estimate expressed as percentage of σ^2_{plot} [1]
σ^2_L	1,381	24.1
$\sigma^2_{R(L)}$	−1	0
σ^2_Y	−77	0
σ^2_{YL}	558	9.7
$\sigma^2_{YR(L)}$	9	.2
σ^2_V	44	.8
σ^2_{VL}	18	.3
σ^2_{VY}	−9	0
σ^2_{VYL}	72	1.2
$\sigma^2_{VR(L)}$	−21	0
$\sigma^2_{VYR(L)}$	−15	0
σ^2_{CL}	12	.2
σ^2_{CY}	1,371	23.9
σ^2_{CYL}	1,368	23.8
σ^2_{CV}	13	.2
σ^2_{CVL}	−16	0
σ^2_{CVY}	47	.8
σ^2_{CVYL}	333	5.8
Error (σ^2)	514	9.0
σ^2_{plot} [2]	5,740	100.0
ΣC_i^2	−406	

[1] $\sigma^2_{plote}=1,381+558+9 \ldots +514$.
[2] Negative components were assumed to estimate zero.

TABLE 5.—*Average coumestrol content of alfalfa harvested at 1/10-bloom stage by varieties (3 cuttings and 7 locations) and by cuttings (5 varieties and 7 locations)*

Item	1960	1961	Average
VARIETIES	*P.p.m.*	*P.p.m.*	*P.p.m.*
Buffalo	57. 7	60. 8	59. 2
Du Puits	52. 9	48. 4	50. 7
Lahontan	71. 9	65. 4	68. 7
Ranger	62. 9	58. 0	60. 4
Vernal	53. 7	52. 4	53. 0
CUTTINGS			
1st	45. 0	107. 1	76. 0
2d	56. 9	17. 0	37. 0
3d	77. 6	46. 9	62. 3
Average	59. 8	57. 0	58. 4

In 1960, as shown in table 6, coumestrol content was lowest in the first cutting in Iowa, Kansas, and North Carolina; highest in the first cutting in California; and not greatly different among cuttings in Nebraska, Pennsylvania, and Utah. In 1961, coumestrol content was consistently high at the first cutting and low at the second cutting at all locations except California.

TABLE 6.—*Average coumestrol content (p.p.m.) of 5 alfalfa varieties harvested at 1/10-bloom stage by cuttings, locations, and years*

Cuttings	California		Iowa		Kansas		Nebraska	
	1960	1961	1960	1961	1960	1961	1960	1961
1st	31. 1	5. 7	98. 4	172. 2	10. 3	247. 1	58. 5	94. 2
2d	16. 6	3. 3	124. 2	16. 6	40. 7	14. 7	54. 7	13. 2
3d	. 2	6. 0	217. 6	123. 8	70. 3	45. 6	55. 5	19. 3
Average of 3 cuttings	15. 9	5. 0	146. 7	104. 2	40. 4	102. 4	56. 2	42. 2
4th	1. 4	12. 9	------	------	41. 3	35. 5	------	------
5th	13. 5	------	------	------	------	------	------	------
Average of all cuttings	12. 6	7. 0	146. 7	104. 2	40. 6	85. 7	56. 2	42. 2
	North Carolina		Pennsylvania		Utah		7 locations	
1st	14. 8	48. 3	98. 5	132. 8	3. 7	49. 3	45. 0	107. 1
2d	83. 4	25. 2	79. 0	41. 9	0	4. 3	56. 9	17. 0
3d	97. 0	43. 7	96. 4	78. 1	6. 4	12. 2	77. 6	46. 9
Average of 3 cuttings	65. 0	39. 0	91. 3	84. 2	3. 4	21. 9	59. 8	57. 0
4th	47. 1	46. 0	------	------	------	------	------	------
Average of all cuttings	60. 6	40. 8	91. 3	84. 2	3. 4	21. 9	------	------

Location averages (p.p.m.) for coumestrol content of three cuttings were lowest in California and Utah, averaging 10.4 and 12.6, respectively (table 5). Highest values were obtained in Iowa, which averaged 125.4. Coumestrol contents of forage were intermediate in Nebraska (49.2), North Carolina (52.0), Kansas (71.4), and Pennsylvania (87.7). Forage from the two first cuttings in Kansas differed tremendously and caused marked differences between years at that location.

Coumestrol averages of three cuttings for 1960 and 1961 were 59.8 and 57.0, respectively as shown in table 7 (lower right), but the year×location interaction was highly significant (table 3). The latter resulted from coumestrol content being lower in 1960 than 1961 in Kansas and Utah, whereas it was higher in 1960 at other locations. The high average coumestrol in Kansas and Utah for 1961, as compared with 1960, was largely due to high levels in the first cutting in 1961.

Cuttings Not Common to All Locations and Data for Individual Locations

Coumestrol determinations for the fourth cutting are given in table 7 for those locations where the season permitted a fourth crop. Generally, content of the fourth cutting was similar to that of the third at the respective locations. Average coumestrol content for the fifth cutting in California in 1960 was 13.5 p.p.m. (table A1) and similar to the average of that location for the first three cuttings. Data for individual locations are given in tables A1–A7 in the appendix for each character studied.

Relationship of Coumestrol Content to Other Chemical Constituents

Averages for chemical constituents determined by feed analysis (1960 samples only) are summarized by varieties, cuttings, and locations in tables 8 and 9 and by locations in tables A1–A7. Mean squares for coumestrol content and regressions of coumestrol with other constituents from the combined statistical analysis over locations are presented in table A8 and for analysis of individual locations in tables A9–A15. Sums of squares due to regression on coumestrol content obtained from the combined analysis (table A8) are summarized in table 10 as percentages. A regression analysis of Utah data was not computed because some coumestrol samples for the third cutting in 1960 were lost. However, Utah data were subjected to an analysis of variance (table A15) and were included in the combined regression analysis.

A correlation coefficient is regarded as significant if the sum of squares due to regression (r^2) was significant at the 5-percent level when coumestrol was used either as the "x" or "y" variable.

Association With Protein.—Coumestrol and protein contents were positively and signficantly correlated only for the cutting×location source of variance (table 10). This suggested that those environmental or management factors peculiar to individual cuttings at individual locations tended to affect coumestrol and protein contents similarly.

At individual locations protein content was also positively correlated with coumestrol content for the cutting×variety interaction. In Kansas (table A3) this appeared to be due primarily to a high level of both constituents in Du Puits forage at the second cutting and in Lahontan forage at the third cutting. In Iowa (table A2) coumestrol and protein contents were lowest at the first cutting and increased in successive harvests, contributing to a positive association for cuttings (table A10).

Association With Crude Fiber.—As coumestrol increased, crude fiber also tended to increase slightly, but the only significant sources were the variety×location and cutting×variety interactions (table 10).

At individual locations crude fiber content was positively correlated with coumestrol content for varieties and cuttings in Iowa and Kansas, respectively (tables A10 and A11). Lahontan had high fiber and coumestrol contents in Iowa (table A2). First-cutting forage in Kansas was low in both fiber and coumestrol (table A3).

Association With Ash.—Coumestrol content was strongly correlated negatively with ash content of forage for locations and cuttings, but not for other sources of variation (table 10).

Ash content was negatively correlated with coumestrol for cuttings in Kansas and for varieties in North Carolina (tables A11 and A13). The third cutting in Kansas was highest in coumestrol and lowest in ash (table A3). Forage of Lahontan and Du Puits in North Carolina was higher in coumestrol and lower in ash than other varieties (table A5).

Association With Fat.—Coumestrol and fat contents were not significantly associated for any of the sources of variance in the combined analysis (table 10). However, in Iowa and Nebraska coumestrol and fat were positively correlated for the cuttings source of variance (tables A10 and A12). Iowa forage was high in coumestrol and fat at the third cutting (table A2), whereas Nebraska forage was high in both constituents at the first cutting and low at the second cutting (table A4).

Association With Nitrogen-Free Extract.—Coumestrol content was negatively and significantly correlated with nitrogen-free extract for variety×location and cutting×variety sources of variance (table 10). Significant negative correlations for some sources of variation were also obtained at all locations except North Carolina and California (tables A9–A14). Sources with significant correlations were either variety or the cutting×variety interaction. In California a positive association was obtained for the variety source of variation (table A9).

Relationship of Coumestrol Content to Plant Height, Defoliation, and Maturity

Data for plant height, defoliation, and maturity are given according to location, cutting, variety, and year in tables A1–A7. Averages over years and locations are presented in table 11. Results of the combined and individual analyses for height and defoliation are shown in tables A16–A20. Sums of squares due to regression on coumestrol content for height and defoliation are summarized as percentages in table 12.

TABLE 7.—*Average coumestrol content (p.p.m.) of alfalfa by cuttings, varieties, localities, and years*

Variety	California		Iowa		Kansas		Nebraska		North Carolina		Pennsylvania		Utah		7 locations		
	1960	1961	1960	1961	1960	1961	1960	1961	1960	1961	1960	1961	1960	1961	1960	1961	2 years
1ST CUTTING																	
Buffalo	30.5	5.0	126.8	237.0	10.5	261.0	63.5	109.0	13.0	56.0	160.3	134.0	3.8	47.0	58.3	121.3	89.8
Du Puits	10.8	1.0	103.0	107.0	8.3	186.5	43.5	24.8	12.0	47.8	74.5	149.3	3.5	55.8	36.5	81.7	59.1
Lahontan	51.8	13.3	98.8	257.0	9.5	284.0	66.0	101.5	16.5	60.0	62.8	146.0	5.0	40.5	44.3	128.9	86.6
Ranger	35.5	4.0	97.8	136.0	12.3	205.8	72.8	118.5	19.3	35.8	125.0	97.8	3.5	53.8	52.3	105.9	79.1
Vernal	26.8	5.3	65.8	123.8	11.0	208.3	46.5	117.0	13.0	42.0	69.8	136.8	2.8	49.3	33.6	97.5	65.6
Average	31.1	5.7	98.4	172.2	10.3	247.1	58.5	94.2	14.8	48.3	98.5	132.8	3.7	49.3	45.0	107.1	76.0
2D CUTTING																	
Buffalo	15.5	5.8	109.3	15.3	33.5	13.8	60.5	15.8	61.8	40.5	72.0	28.3	0	3.5	50.4	17.5	33.9
Du Puits	16.0	2.8	116.0	19.5	64.8	16.0	50.5	1.8	76.0	19.8	70.0	48.3	0	3.5	56.2	15.9	36.1
Lahontan	25.5	2.8	140.5	16.5	51.0	16.5	47.8	16.3	95.0	21.8	89.3	41.8	0	3.8	64.1	17.0	40.6
Ranger	13.3	3.3	137.8	18.5	26.8	16.8	81.8	18.0	91.0	20.3	88.0	49.0	0	4.8	62.6	18.6	40.6
Vernal	12.8	1.8	117.5	13.3	27.3	10.5	32.8	14.0	93.0	23.5	75.5	42.3	0	6.0	51.3	15.9	33.6
Average	16.6	3.3	124.2	16.6	40.7	14.7	54.7	13.2	83.4	25.2	79.0	41.9	0	4.3	56.9	17.0	37.0

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3D CUTTING

Buffalo	0	7.5	169.8	115.0	57.8	46.0	52.3	18.8	99.3	46.0	66.8	59.0	5.0	13.0	64.4	43.6	54.0
Du Puits	0	2.0	146.0	150.8	71.8	44.5	35.5	15.8	133.5	58.5	72.0	54.0	4.0	8.0	66.1	47.6	56.9
Lahontan	.8	5.0	308.3	111.5	97.8	53.0	89.5	21.8	99.3	37.8	151.0	115.5	5.0	7.8	107.4	50.3	78.8
Ranger	0	9.0	215.0	129.3	69.3	49.5	52.3	23.0	69.8	36.5	104.0	86.8	7.0	11.3	73.9	49.3	61.6
Vernal	0	6.5	249.0	112.5	54.8	34.8	47.8	17.3	83.0	39.8	88.3	75.0	11.0	20.8	76.3	43.8	60.0
Average	.2	6.0	217.6	123.8	70.3	45.6	55.5	19.3	97.0	43.7	96.4	78.1	6.4	12.2	77.6	46.9	62.3

AVERAGE OF 3 CUTTINGS

Buffalo	15.3	6.1	135.3	122.4	33.9	106.9	58.8	47.8	58.0	47.5	99.7	73.8	2.9	21.2	57.7	60.8	59.2
Du Puits	8.9	1.9	121.7	92.4	48.3	82.3	43.2	14.1	73.8	42.0	72.2	83.8	2.5	22.4	52.9	48.4	50.7
Lahontan	26.0	7.0	182.5	128.3	52.8	117.8	67.8	46.5	70.3	39.8	101.0	101.1	3.3	17.3	71.9	65.4	68.7
Ranger	16.3	5.4	150.2	94.6	36.1	120.7	68.9	53.2	60.0	30.8	105.7	77.8	3.5	23.3	62.9	58.0	60.4
Vernal	13.2	4.5	144.1	83.2	31.0	84.5	42.3	49.4	63.0	35.1	77.8	84.7	4.6	25.3	53.7	52.4	53.0
Average	15.9	5.0	146.7	104.2	40.4	102.4	56.2	42.2	65.0	39.0	91.3	84.2	3.4	21.9	59.8	57.0	58.4

4TH CUTTING

Buffalo	4.0	14.0			36.3	29.0			42.8	43.5							
Du Puits	0	10.8			44.8	27.0			40.3	40.8							
Lahontan	0	9.8			45.0	45.0			61.5	65.0							
Ranger	0	13.8			35.8	40.3			44.3	42.3							
Vernal	2.8	16.0			44.5	36.3			46.5	38.3							
Average	1.4	12.9			41.3	35.5			47.1	46.0							

TABLE 8.—*Chemical composition of alfalfa by cuttings and varieties averaged over 7 locations, 1960*

Variety	Coumestrol	Protein	Crude fiber	Ash	Fat	N-free extract
	1ST CUTTING					
	P.p.m.	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Buffalo	58.3	19.8	27.1	10.3	3.2	39.6
Du Puits	36.5	20.0	25.6	10.4	3.3	40.7
Lahontan	44.3	19.8	26.6	10.2	3.4	40.2
Ranger	52.3	19.9	26.0	10.3	3.3	40.5
Vernal	33.6	19.8	26.3	10.1	3.3	40.5
Average	45.0	19.8	26.3	10.2	3.3	40.3
	2D CUTTING					
Buffalo	50.4	20.6	25.6	10.2	3.2	40.4
Du Puits	56.2	20.9	26.3	9.9	3.3	39.6
Lahontan	64.1	20.3	25.9	9.8	3.3	40.8
Ranger	62.6	20.9	25.9	10.0	3.1	40.2
Vernal	51.3	21.1	27.1	9.7	3.4	38.8
Average	56.9	20.8	26.2	9.9	3.2	39.9
	3D CUTTING					
Buffalo	64.4	19.8	26.7	9.7	3.1	40.7
Du Puits	66.1	20.0	26.3	9.5	3.2	40.8
Lahontan	107.4	20.0	27.4	9.5	3.1	40.1
Ranger	73.9	20.3	26.2	9.5	3.2	40.8
Vernal	76.3	20.9	26.0	9.5	3.3	40.5
Average	77.6	20.2	26.5	9.5	3.2	40.6
	AVERAGE OF 3 CUTTINGS					
Buffalo	57.7	20.1	26.5	10.1	3.2	40.2
Du Puits	52.9	20.3	26.1	9.9	3.3	40.4
Lahontan	71.9	20.0	26.6	9.8	3.3	40.4
Ranger	62.9	20.4	26.0	9.9	3.2	40.5
Vernal	53.7	20.6	26.5	9.8	3.3	39.9
Average	59.8	20.3	26.3	9.9	3.2	40.3

Association With Plant Height.—Coumestrol and plant height were significantly correlated, positively for cuttings, cuttings×locations, varieties×locations, and negatively for cuttings×years, cuttings×varieties, and cuttings×varieties×locations (tables 12 and A16). The largest estimated variance components for coumestrol were for the location, cutting×year, and cutting×year×location (table 4), but only the cutting×year source was correlated with height. The relationship between coumestrol content and height was also inconsistent at individual locations (tables A17–A19).

TABLE 9.—*Chemical composition of alfalfa by locations and cuttings averaged over 5 varieties, 1960*

Location	Coumestrol (p.p.m.)				Protein (percent)				Crude fiber (percent)			
	1st cutting	2d cutting	3d cutting	Average	1st cutting	2d cutting	3d cutting	Average	1st cutting	2d cutting	3d cutting	Average
California	31.1	16.6	0.2	15.9	20.8	22.1	20.3	21.1	25.9	27.5	26.9	26.8
Iowa	98.4	124.2	217.6	146.7	18.5	19.4	22.5	20.1	31.2	26.5	26.3	28.0
Kansas	10.3	40.7	70.3	40.4	19.5	19.2	19.6	19.4	25.1	27.6	28.2	27.0
Nebraska	58.5	54.7	55.5	56.2	17.4	17.9	18.8	18.0	31.5	32.2	31.5	31.7
North Carolina	14.8	83.4	97.0	65.0	22.3	28.1	24.5	25.0	22.7	19.1	23.1	21.6
Pennsylvania	98.5	79.0	96.4	91.3	21.5	18.4	18.2	19.4	29.9	30.6	25.5	28.7
Utah	3.7	0	6.4	3.4	18.9	20.2	17.4	18.9	17.8	19.6	24.4	20.6
Average	45.0	56.9	77.6	59.8	19.8	20.8	20.2	20.3	26.3	26.2	26.6	26.3
	Ash (percent)				Fat (percent)				N-free extract (percent)			
California	10.4	10.7	10.3	10.5	3.0	2.6	2.8	2.8	40.0	37.1	39.7	38.9
Iowa	7.9	7.3	7.7	7.6	2.9	2.9	3.2	3.0	39.4	43.9	40.3	41.2
Kansas	11.8	11.3	9.9	11.0	3.5	3.2	3.2	3.3	40.1	38.6	39.1	39.3
Nebraska	9.5	9.8	9.2	9.5	3.4	2.7	3.0	3.0	38.2	37.5	37.5	37.7
North Carolina	10.1	9.8	9.5	9.8	3.5	4.0	3.4	3.6	41.4	39.1	39.4	40.0
Pennsylvania	10.4	8.8	8.7	9.3	2.9	3.1	3.2	3.1	35.2	39.0	44.4	39.6
Utah	11.6	11.6	11.3	11.5	3.9	4.2	3.5	3.9	47.7	44.4	43.4	45.2
Average	10.2	9.9	9.5	9.9	3.3	3.2	3.2	3.2	40.3	39.9	40.5	40.3

TABLE 10.—*Percentage of reductions in sums of squares due to regression* (r^2) *on coumestrol content for certain sources of variation in analysis of variance of chemical constituents in 1960 forage.*[1] *(See table A8 for mean squares.)*

Source of variation	Protein	Crude fiber	Ash	Fat	N-free extract
Locations (L)	+0.7	+16.8	−89.7†† **	−12.1	−3.6
Varieties (V)	−42.6	+21.8	−7.2	−23.5	+17.9
V×L	0	+15.9† *	−.1	+8.7	−24.8† **
Cuttings (C)	+5.0	+51.7	−97.2*	−99.3	+31.4
C×L	+50.1†† **	−10.0	+1.0	+9.6	−5.9
C×V	+11.5	+24.6†	0	−23.4	−31.6†
C×V×L	+1.5	+.4	−1.7	−7.8	−1.1

[1] + or − = sign of r; † and †† = significant at 5- and 1-percent levels, respectively, with coumestrol used as y variable; * and ** = significant at 5- and 1-percent levels, respectively, with coumestrol used as x variable.

Association With Defoliation.—Coumestrol was significantly and positively correlated with defoliation for the following seven sources of variance in the combined analysis: Locations, varieties, varieties × locations, varieties × years, cuttings × years, cuttings × years × locations, and cuttings × varieties × years (table 12). These sources included those found to be major sources of variance in coumestrol (table 4). Defoliation in these experiments probably resulted primarily from leaf and stem diseases, which are most serious under humid conditions. Leafhoppers also might have been a factor.

The first- and third-cutting forages in Utah in 1961 were higher in coumestrol than other cuttings at this location and also had lost more leaves (table A7). Defoliation was not serious for any cuttings in California (table A1). Positive relationships between coumestrol content and defoliation within cuttings are apparent in the 1960 and 1961 data for the first two Kansas cuttings (table A3), for the first and third cuttings in Nebraska (table A4), and for all three cuttings in Pennsylvania (table A6).

Lahontan averaged highest and Du Puits and Vernal lowest in coumestrol content and defoliation (table 11). Ranking of varieties over locations was the same when arranged according to coumestrol content, defoliation score, or expected reaction to foliar diseases.

Association With Maturity.—Maturity scores did not vary greatly among the many samples of forage analyzed for coumestrol. Du Puits averaged a little more mature at harvesttime than the other four varieties and was lowest in coumestrol (table 11). On the other hand, first-cutting forage was scored less mature than either second- or third-cutting forage, but was highest in coumestrol. Range in maturity of samples in this part of the study was relatively narrow, and little information was gained by detailed examination of the maturity score data.

Relationship of Coumestrol Content to Growing Conditions Shortly Before Harvesting

Average soil moisture and sunshine scores and temperatures shortly before harvest, as shown in table 13, failed to show any association between coumestrol content of forage and any environmental conditions estimated. Also, other kinds of data needed for critical determination of these relationships were not obtained. Therefore, statistical data obtained in this part of the investigation were omitted.

TABLE 11.—*Coumestrol content, height, defoliation, and maturity of alfalfa averaged over 2 years at all locations, except Ames, Iowa*

Variety	Coumestrol	Height	Defoliation	Maturity
	1ST CUTTING			
	P.p.m.	*Inches*	*Score* [1]	*Score* [2]
Buffalo	74.4	25.2	2.0	1.9
Du Puits	51.4	27.6	1.6	2.0
Lahontan	71.4	25.3	2.1	1.8
Ranger	72.8	24.2	2.0	1.9
Vernal	60.7	25.6	1.9	1.9
Average	66.1	25.6	1.9	1.7
	2D CUTTING			
Buffalo	29.2	23.7	1.5	2.0
Du Puits	30.8	24.4	1.0	2.2
Lahontan	34.3	23.3	1.4	1.9
Ranger	34.4	23.5	1.2	1.9
Vernal	28.3	22.6	1.0	1.9
Average	31.4	23.5	1.2	2.0
	3D CUTTING			
Buffalo	39.3	25.0	2.2	2.1
Du Puits	41.6	25.8	1.9	2.4
Lahontan	57.0	24.1	2.8	2.2
Ranger	43.2	23.5	2.3	2.1
Vernal	39.9	23.1	2.0	2.1
Average	44.2	24.3	2.2	2.2
	AVERAGE OF 3 CUTTINGS			
Buffalo	47.6	24.6	1.9	2.0
Du Puits	41.3	25.9	1.5	2.2
Lahontan	54.2	24.2	2.1	2.0
Ranger	50.1	23.7	1.8	2.0
Vernal	43.0	23.8	1.6	2.0
Average	47.2	24.4	1.8	2.0

[1] From 0 to 7, representing 0- to 70-percent or greater defoliation, respectively.
[2] 1=late-bud stage, 2=1/10-bloom stage, 3=1/2-bloom, and 4=full bloom.

TABLE 12.—*Percentage of reduction in sums of squares due to regression* (r^2) *on coumestrol content for certain sources of variation in combined analyses of variance for height and defoliation.*[1] *(See table A16.)*

Source of variation	Height	Defoliation
Locations (L)	−13.0	+81.4†† *
Replications in locations (R (L))	−.6	−.1
Years (Y)×L	−36.9	−8.0
Y×R (L)	0	0
Varieties (V)	−24.3	+99.0†† **
V×L	+12.6*	+30.4†† *
V×Y	+2.8	+76.4 *
V×Y×L	−1.5	+.2
V×R (L)	+.1	+.1
V×Y×R (L)	−.3	−2.6
Cuttings (C)	+100.0†† **	+28.4
C×L	+44.3† *	+2.6
C×Y	−97.9†† **	+100.0†† **
C×Y×L	−10.4	+40.7† *
C×V	−31.8*	+10.5
C×V×L	−16.2†† **	+4.7
C×V×Y	+5.6	+36.7*
C×V×Y×L	+1.8	+6.7

[1] + or − =sign of r; † and ††=significant at 5- and 1-percent levels, respectively, with coumestrol as y variable; * and ** =significant at 5- and 1-percent levels, respectively, with coumestrol as x variable.

TABLE 13.—*Soil moisture, sunshine, and temperatures* [1] *shortly before harvesting alfalfa samples at 1/10-bloom stage*

Location and cutting	1960				1961			
	Soil moisture	Sunshine	Maximum temperature	Minimum temperature	Soil moisture	Sunshine	Maximum temperature	Minimum temperature
	Score	*Score*	*° F.*	*° F.*	*Score*	*Score*	*° F.*	*° F.*
California:								
1st	3.0	1.0	73.0	44.0	2.7	3.0	77.6	52.4
2d	3.0	2.0	79.0	51.0	3.0	1.8	89.8	54.8
3d	3.0	1.0	92.0	52.0	3.0	1.0	92.6	53.4
4th	3.0	1.0	91.2	51.6	2.7	1.0	89.8	51.6
5th	3.0	1.4	92.8	55.4	----------	----------	----------	----------
Iowa:								
1st	3.0	----------	77.0	56.0	3.0	----------	89.0	68.0
2d	2.0	----------	91.0	68.0	3.0	----------	76.0	61.0
3d	3.0	----------	90.0	67.0	2.0	----------	86.0	62.0
Kansas:								
1st	3.0	2.0	74.2	52.6	3.0	1.6	86.8	59.4
2d	3.0	1.8	89.8	68.2	3.0	1.0	96.4	70.8
3d	3.0	1.2	87.6	68.4	3.0	1.6	92.2	72.6
4th	3.0	2.2	83.2	53.0	3.0	1.6	83.8	53.8
Nebraska:								
1st	3.0	1.2	89.8	60.4	3.0	3.2	87.6	66.8
2d	3.0	4.0	84.8	63.2	3.0	1.6	89.2	75.6
3d	3.0	2.2	84.4	65.8	3.0	4.0	81.4	64.8
North Carolina:								
1st	2.0	1.0	74.0	42.0	2.0	4.0	86.0	66.0
2d	3.0	2.0	81.0	62.0	2.0	2.0	86.0	59.0
3d	3.0	2.0	92.0	63.0	2.0	1.0	88.0	64.0
4th	3.0	2.0	82.0	64.0	2.0	1.0	90.0	64.0
Pennsylvania:								
1st	3.0	2.0	67.0	52.0	2.0	1.0	80.0	58.0
2d	3.0	2.0	82.0	60.0	3.0	2.0	82.0	68.0
3d	1.0	1.0	82.0	63.0	2.0	1.0	88.0	62.0
Utah:								
1st	1.0	2.0	77.0	53.0	3.0	1.0	80.0	45.0
2d	2.0	1.0	98.0	59.0	2.8	3.0	88.0	58.0
3d	1.7	1.0	87.0	48.0	3.0	2.0	82.0	55.0

[1] For method of recording data, see p. 5.

VARIATION IN COUMESTROL CONTENT AS AFFECTED BY GROWTH STAGE

Materials and Methods

In a separate experiment, broadcast plots of Buffalo and Ranger, 5 feet wide and 20 feet long, were planted in four replications at Lincoln, Nebr., and Davis, Calif., to study the effect of growth stage on coumestrol content of alfalfa. To avoid residual effects of different harvest dates on performance in subsequent cuttings, separate plots were established for each cutting. Each plot was harvested in six parts, the first when growth was 10 inches high. The other five parts were harvested successively to obtain samples in the following additional stages of growth: Bud, 1/10 bloom, full bloom, and 10 and 25 days after full bloom. The six growth-stage treatments within variety plots occurred in a fixed order from earliest to latest to permit progressive harvesting of successive growth stages without mechanical difficulties. Thus, each plot was subdivided and sampled only once in a season; plots not receiving a differential cutting treatment were routinely harvested at the 1/10-bloom stage.

Agronomic and coumestrol data were obtained on all plots in 1960 and 1961. In 1961, replication 1 was combined with 2, and 3 with 4 for coumestrol determination. As in the 1/10-bloom phase of the study, only samples obtained in 1960 were subjected to general feed analyses.

Because samples from California and Nebraska differed markedly in average coumestrol content in 1960, a third set of Buffalo and Ranger plots was established at University Park, Pa., in 1961 to obtain samples at the six growth stages.

In other respects, the stage-of-growth investigation was conducted in the same manner as the 1/10-bloom-stage investigation.

Results

Coumestrol, protein, and fiber data obtained in the stage-of-growth phase of the study are given in figures 1–4.

Coumestrol Content

Mean squares for stages of growth and some interactions were significant at the three locations, as shown in table 14. In Nebraska, varieties tended strongly to differ in coumestrol content, but the differences were not significant (table 14 and fig. 2).

At each cutting and location, coumestrol content usually increased with successive stages of growth and reached a maximum 25 days after full bloom, the most mature stage harvested (fig. 1). Coumestrol content averaged over cuttings increased with successive stages of growth in Nebraska and Pennsylvania (fig. 2). However, in California it was highest 10 days after full bloom. It was consistently lower in California than in Nebraska or Pennsylvania (figs. 1 and 2). Coumestrol content averaged over stages of growth for three cuttings at each location is given in table 15.

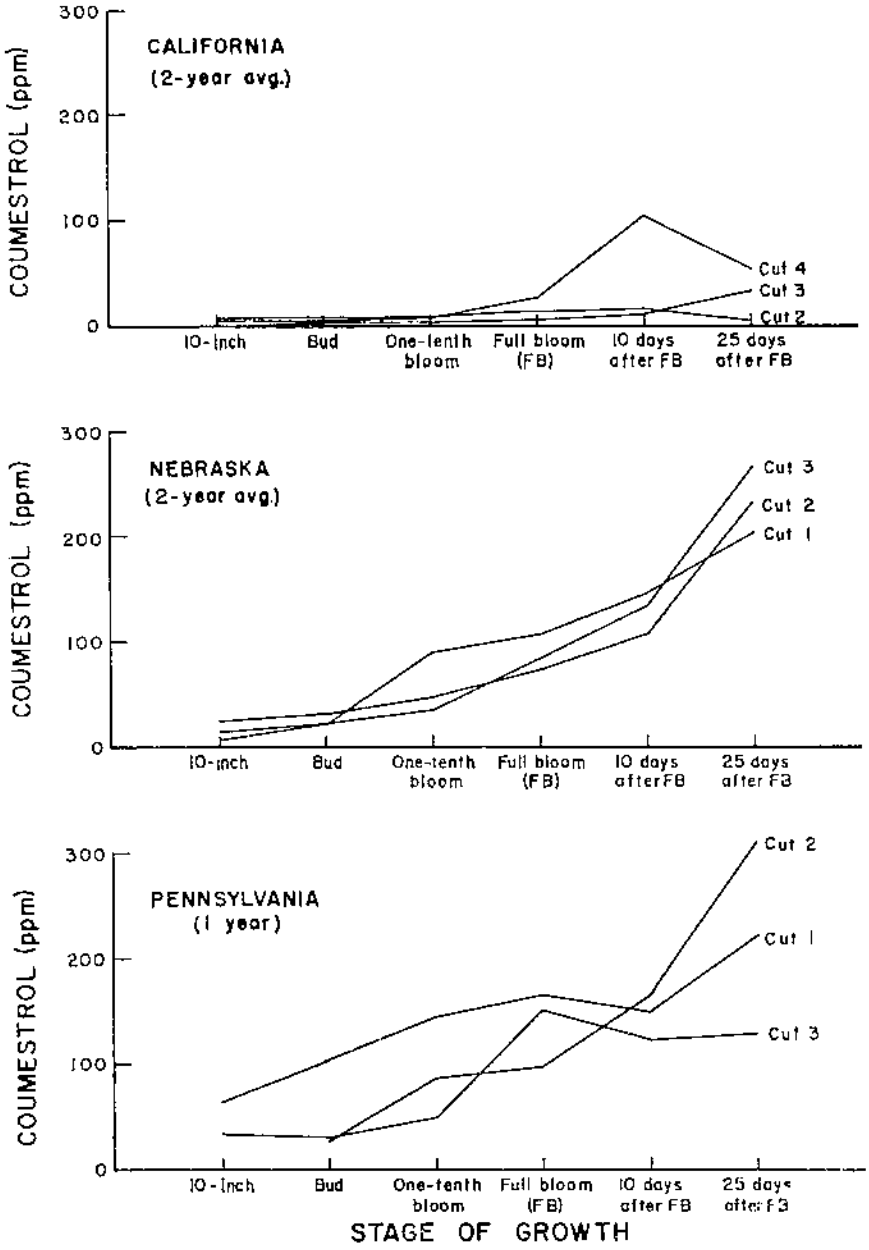

FIGURE 1.—Coumestrol content of Buffalo and Ranger alfalfa at six stages of growth and three cuttings averaged over varieties, Davis, Calif., Lincoln, Nebr., and University Park, Pa., 1960 and 1961.

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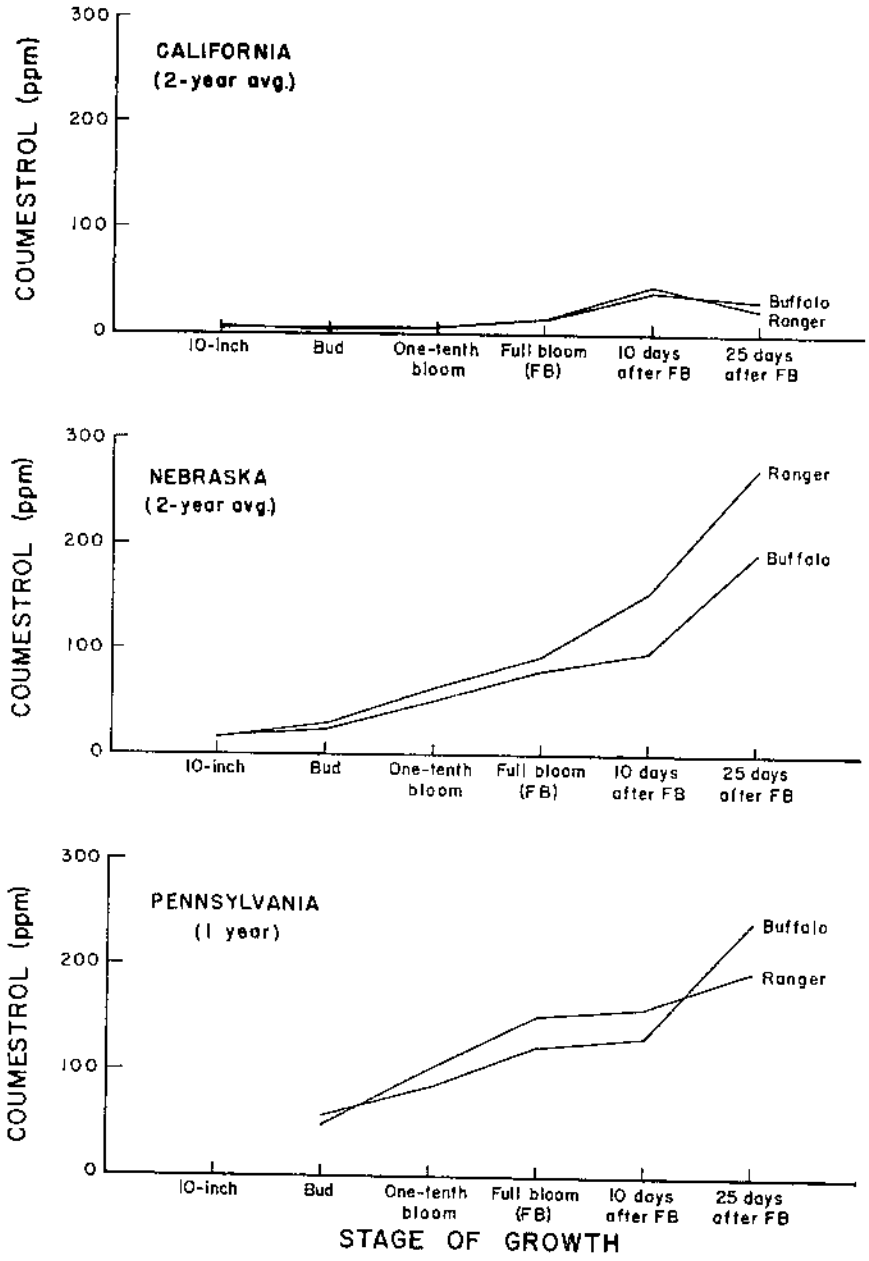

FIGURE 2.—Coumestrol content of Buffalo and Ranger alfalfa at six stages of growth averaged over cuttings, Davis, Calif., Lincoln, Nebr., and University Park, Pa., 1960 and 1961.

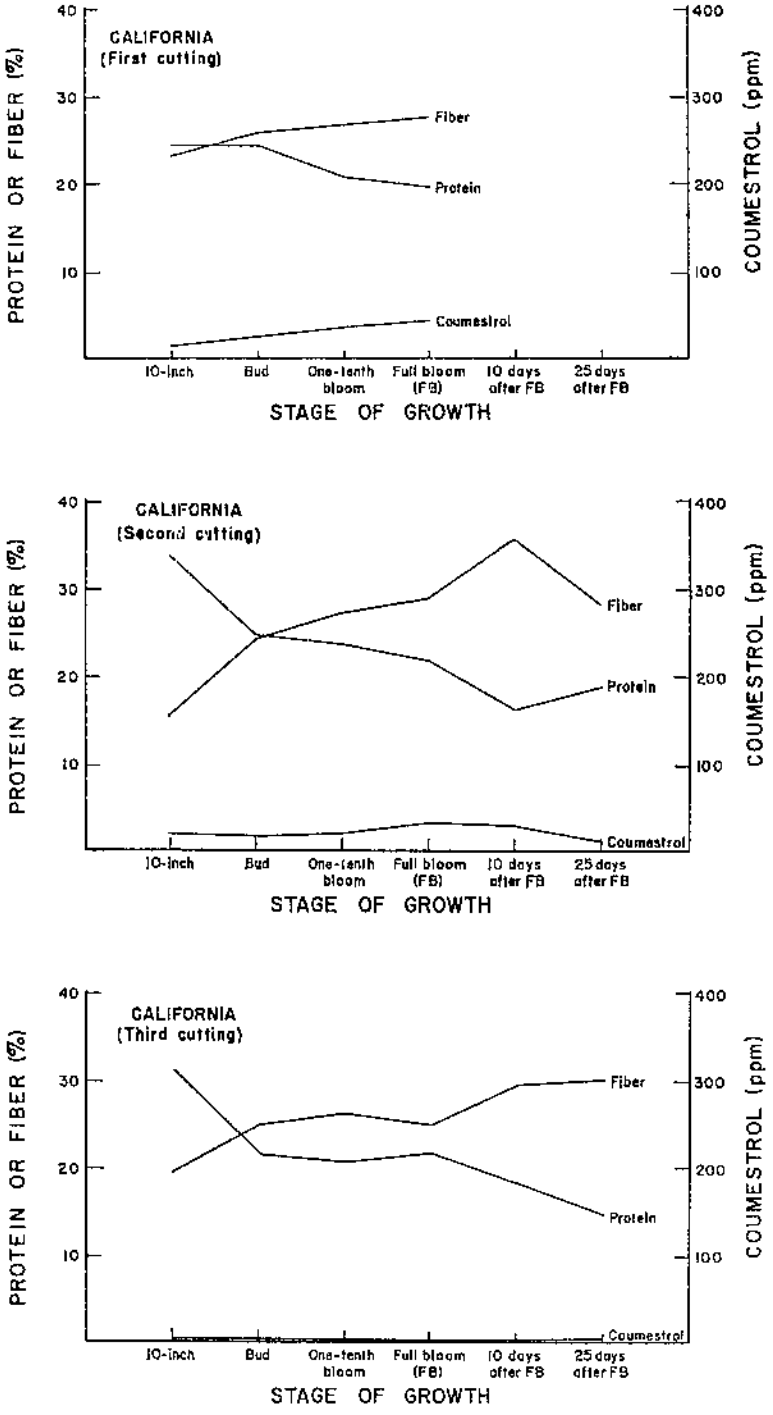

FIGURE 3.—Coumestrol, protein, and fiber contents of Buffalo and Ranger alfalfa at six stages of growth averaged over varieties, Davis, Calif., 1960.

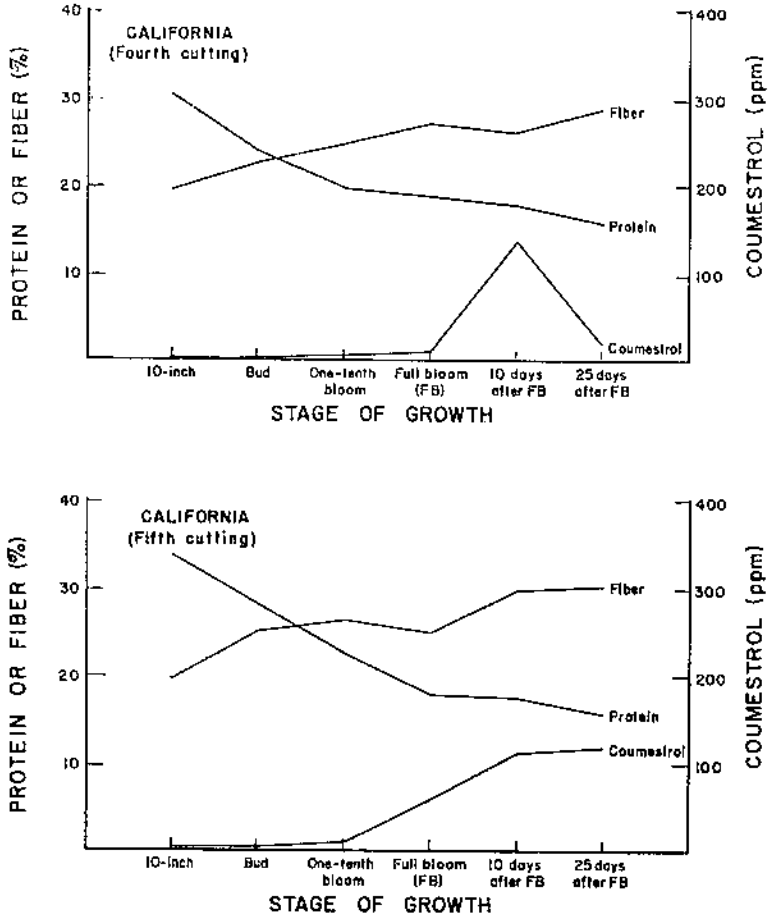

FIGURE 3.—Coumestrol, protein, and fiber contents of Buffalo and Ranger alfalfa at six stages of growth averaged over varieties, Davis, Calif., 1960—Continued.

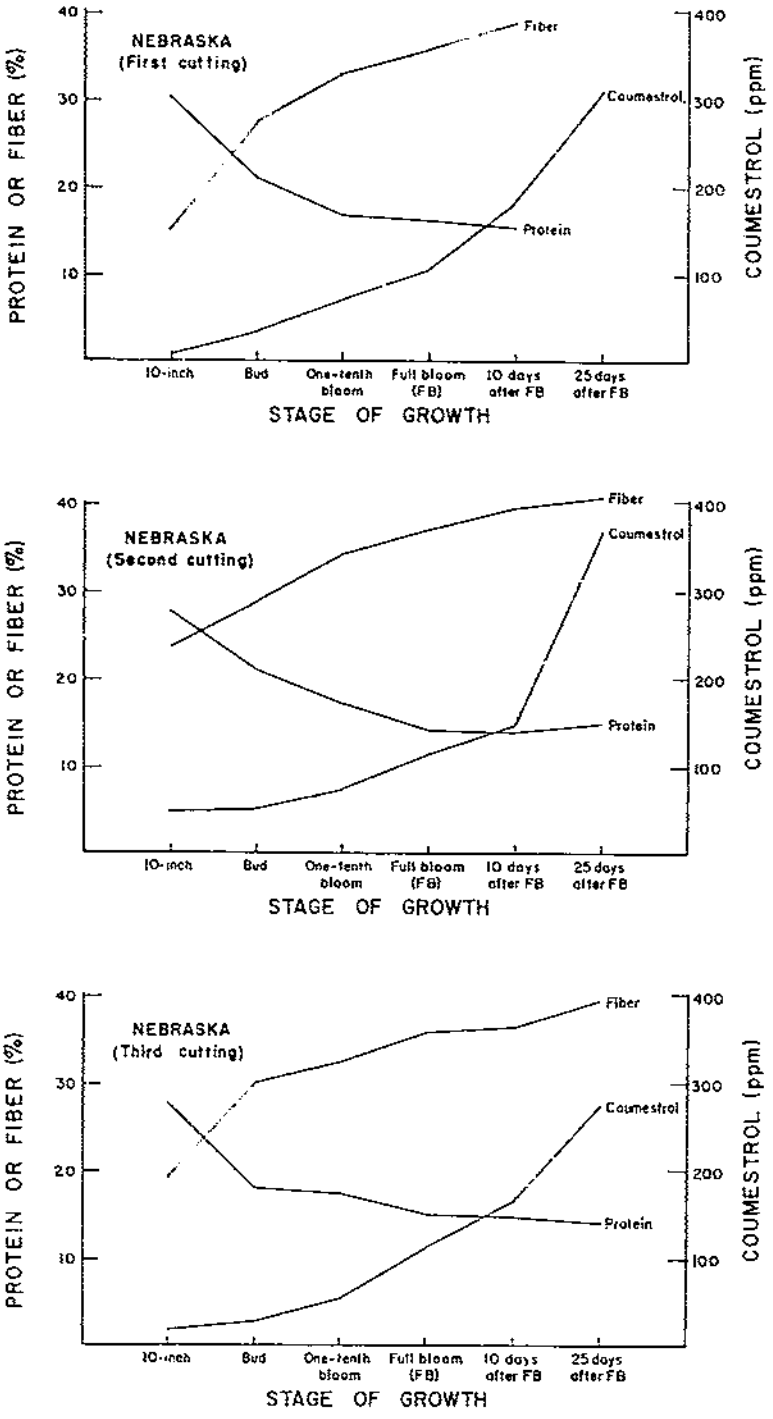

FIGURE 4.—Coumestrol, protein, and fiber contents of Buffalo and Ranger alfalfa at six stages of growth averaged over varieties, Lincoln, Nebr., 1960.

TABLE 14.—*Mean squares from analyses of variance of coumestrol content of Buffalo and Ranger alfalfa harvested at 6 stages of growth and 3 locations* [1]

Source	d.f. for California and Nebraska	California [2] (1960 and 1961)	Nebraska (1960 and 1961)	d.f. for Pennsylvania	Pennsylvania [3] (1961)
Total	143			58	
Replications (R)	1	149	566	1	454
Varieties (V)	1	0	27, 542	1	126
Error a	1	25	226	1	1, 591
Years (Y)	1	888	115, 458		
Y×V	1	18	5, 669		
Error b	2	61	572		
Cuttings (C)	2	9, 286	1, 035	2	18, 242
C×V	2	58	2, 974	2	2, 563
C×Y	2	3, 038*	12, 165*		
C×V×Y	2	31	270		
Error c	8	86	1, 559		
Stages (S)	5	6, 065	158, 694*	4	46, 622**
S×V	5	41	6, 810*	4	2, 863
S×Y	5	2, 389**	19, 425**		
S×V×Y	5	15	1, 302		
S×C	10	3, 900*	3, 851	8	9, 217**
S×C×V	10	63	1, 167	8	897
S×C×Y	10	1, 174**	6, 407**		
S×C×V×Y	10	26	462		
R×S×V				4	498
Error d [4]		118	927		461

[1] * and **=significant at 5- and 1-percent levels, respectively.
[2] Computed with 2d, 3d, and 4th cuttings because data from 1st cutting were incomplete.
[3] 10-inch stage not included in analysis because data were incomplete.
[4] Degrees of freedom for error were as follows: California 57, Nebraska 59, and Pennsylvania 23.

TABLE 15.—*Coumestrol content of Buffalo and Ranger alfalfa at 3 locations and 3 cuttings averaged over stages of growth, 1960 and 1961* [1]

Location	1st	2d	3d	Average
	1960 CUTTINGS			
	P.p.m.	*P.p.m.*	*P.p.m.*	*P.p.m.*
California	16. 8	0. 5	27. 8	15. 0
Nebraska	116. 3	131. 9	108. 1	118. 8
Pennsylvania				
	1961 CUTTINGS			
California	3. 6	17. 0	39. 3	20. 0
Nebraska	72. 9	39. 0	74. 5	62. 1
Pennsylvania	156. 4	136. 6	97. 0	130. 0

[1] 2d, 3d, and 4th cuttings for California are shown under 1st, 2d, and 3d cuttings, respectively, because all stages were not obtained in the 1st cutting of 1960.

The 10 highest coumestrol values, averaged over replications, obtained for any stage of growth within varieties, cuttings, years, and locations are given in table 16. The stage of growth for each was full bloom plus 25 days, except for the lowest value, which was obtained at full bloom plus 10 days.

TABLE 16.—*Highest coumestrol values obtained for Buffalo and Ranger alfalfa at 3 cuttings, 2 years, and 3 locations*

Coumestrol (p.p.m.)	Variety	Cutting	Year	Location
429.2	Ranger	2d	1960	Nebraska.
349.0	do	1st	1960	Do.
336.8	do	3d	1960	Do.
323.0	Buffalo	2d	1961	Pennsylvania.
318.0	Ranger	3d	1961	Nebraska.
304.0	Buffalo	2d	1960	Do.
295.5	Ranger	2d	1961	Pennsylvania.
264.2	Buffalo	1st	1960	Nebraska.
254.0	do	1st	1961	Pennsylvania.
245.2	Ranger	3d	1960	Nebraska.

Relationship of Coumestrol Content to Other Chemical Constituents

In Nebraska, increases in coumestrol content associated with successive stages of growth were accompanied by increases in crude fiber and decreases in protein contents (fig. 4). These associations were consistent for individual cuttings as well as for cuttings combined. The slight increase in protein content at the last growth stage in the second cutting was due to axillary bud growth. In California (fig. 3), generally coumestrol was negatively associated with protein content and positively associated with fiber content, but the associations were less consistent than in Nebraska. Also, fiber and coumestrol contents did not reach as high levels and protein as low levels as in Nebraska.

Coumestrol content was negatively associated with ash content in Nebraska and California.

At both locations, nitrogen-free extract increased from the 10-inch to the bud stage. In Nebraska, nitrogen-free extract was fairly constant after the bud stage. In California, nitrogen-free extract content was fairly constant from the bud through the full-bloom plus 10-day stage, then increased at the last stage. Thus, there was no consistent relationship between nitrogen-free extract and coumestrol contents.

Relationship of Coumestrol Content to Plant Height and Defoliation

Height increased with successive stages of growth at each of the three locations. Increases in height were greatest from the 10-inch to the $\frac{1}{10}$-bloom stages. Thus, height and coumestrol content tended to be associated.

Coumestrol content and defoliation were positively associated in Nebraska. Defoliation gradually increased from zero at the 10-inch stage to about 70 percent at full bloom plus 25 days in all varieties, cuttings, and years in Nebraska.

EFFECT OF LIGHT INTENSITY ON COUMESTROL CONTENT

This was a preliminary experiment to determine whether shading of alfalfa might increase coumestrol content. The study was conducted in 1961 at Logan, Utah, after learning that the 1960 samples from Utah and California were extremely low in coumestrol content.

Materials and Methods

Two plots each of Lahontan and Ranger seeded in the fall of 1959 were shaded individually with cages after the first crop had been harvested. Comparable plots of each variety were left unshaded. This arrangement provided two replications of variety and shading treatments in the second cutting. Frames for cages were 4 feet high, 5 feet wide, and 9½ feet long, and were constructed from ½-inch zinc-coated conduit. The shade cloth covering the frame was of Lumite material, which reduced light intensity by about 72 percent.

Sections of each plot were successively harvested to give the six growth stages (prebud to full bloom+25 days) used in Nebraska, California, and Pennsylvania. Beginning and ending harvest dates were June 30 and August 21, respectively. The procedure for handling samples for coumestrol determination is described on page 6.

Results

Coumestrol content ranged from 3.0 to 12.0 p.p.m., as shown in table 17. Average contents for shaded and unshaded plots, averaged over the six stages of growth, were 7.4 and 5.7 p.p.m., respectively. Analysis of variance indicated that effects of stage of growth, variety, and shading on coumestrol content were not significant.

TABLE 17.—*Coumestrol content of second cutting of Lahontan and Ranger alfalfa grown with and without shade, Logan, Utah, 1961*

Stage of growth and variety	Shaded	Unshaded	Average
	P.p.m.	*P.p.m.*	*P.p.m.*
Prebud:			
Lahontan	4.5	6.0	5.2
Ranger	5.0	6.5	5.8
Average	4.8	6.2	5.5
Early bud:			
Lahontan	8.5	3.0	5.8
Ranger	7.5	3.5	5.5
Average	8.0	3.2	5.6
Early bloom:			
Lahontan	6.0	3.0	4.5
Ranger	7.0	4.0	5.5
Average	6.5	3.5	5.0
Full bloom (FB):			
Lahontan	6.5	3.5	5.0
Ranger	6.5	5.0	5.8
Average	6.5	4.2	5.4
FB+10 days:			
Lahontan	12.0	8.5	10.2
Ranger	3.5	5.5	4.5
Average	7.8	7.0	7.4
FB+25 days:			
Lahontan	10.0	11.5	10.8
Ranger	12.0	8.0	10.0
Average	11.0	9.8	10.4
Grand average:			
Lahontan	7.9	5.9	6.9
Ranger	6.9	5.4	6.2
Average	7.4	5.7	6.5

RELATIONSHIP OF COUMESTROL CONTENT TO FOLIAR DISEASES

Regression analyses showed a consistent positive relationship between coumestrol content and degree of defoliation (p. 18). In controlled environment studies (*27*) coumestrol content was low regardless of the environmental variable, but increased sharply when foliage was infected with either of two pathogens. A cause and effect relationship between foliar diseases and coumestrol content was indicated. In this part of the study coumestrol determinations were made at Brookings, S. Dak.

Effect of Fungicidal Control of Foliar Diseases on Coumestrol Content

Materials and Methods

In 1963, after removal of the second hay crop from a 3-year-old field of Ranger alfalfa at Brookings, a part of the field was divided into eight plots, 10 by 10 feet, and used for an experiment to determine whether control of foliar diseases reduced coumestrol content.

An aqueous solution of Dithane M–45 was applied with a hand sprayer, according to a procedure described by Banttari et al. (*5*), to four plots selected at random. Spray applications were made at 7-day intervals and usually after each rain. They were begun on July 29, when growth of the third crop was 6 inches high, and discontinued on September 12, when the alfalfa was 9 days past full bloom. Spray applications contained 0.56 pound of Dithane M–45 and 50 and 120 ml., respectively, of Tween 21 and Plyac per 100 gallons of water to promote wetting and sticking. Rate of application was 120 gallons of fungicide solution per acre. The four other plots were used as unsprayed checks.

Each of the eight plots was divided into four parts to permit harvesting at four stages of growth—early bud, one-tenth bloom, full bloom, and full bloom plus 10 days. Height of cutting was 2 inches. Leaves and stems were separated, and an aliquot of each sample was taken for moisture determination. Tissue for coumestrol extraction was placed in 4-ounce jars containing 67 ml. of 100-percent methanol. Leaf and stem tissues were extracted for 3 days and aliquots removed for analysis as described by Livingston et al. (*26*). Four separate quantitative determinations were made on the extract for each sample, averaged, and expressed as parts per million on a dry-weight basis as in the entire study.

Results

Abundant inoculum on stubble of the second crop apparently caused heavy foliar infection in untreated plots. The principal leaf pathogens were common leaf spot (*Pseudopeziza medicaginis* (Lib.) Sacc.), rust (*Uromyces striatus* Schroet. var. *medicaginis* (Pass.) Arth.), and *Cercospora* leaf spot (*Cercospora zebrina* Pass.). The last also caused extensive stem blackening beginning about the full-bloom stage. Rust did not become prevalent until the full-bloom stage.

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Defoliation and disease symptoms were much less severe in sprayed than in unsprayed plots, indicating moderately good disease control in sprayed plots. At the full-bloom stage, defoliation in sprayed plots was limited to the lower 5 inches, whereas defoliation occurred in the lower 10 inches of unsprayed plots. Ten days after the full-bloom stage, no further defoliation had occurred in the sprayed plots, but the lower 12 inches of unsprayed plants were completely defoliated. Plant height for the four stages of growth ranged from 18 to 22 inches.

Differences in coumestrol content due to spray treatment, stage of growth, and plant parts were statistically significant. Coumestrol content of leaves and stems in unsprayed alfalfa increased with successive stages of growth, but the greater increase occurred in the stems (fig. 5). The latter appeared to be related to *Cercospora zebrina* infection, which characteristically attacks leaves before stems. Also, defoliation of severely infected leaves might have caused coumestrol content of leaves remaining on the plant to be comparatively constant. Coumestrol content of sprayed plots was relatively low and constant over stages of growth. Coumestrol contents for sprayed and unsprayed plots averaged over stages were 28.9 and 72.9 p.p.m., respectively. On the last sampling date, stems and leaves from sprayed plots averaged 28.6 p.p.m. as compared with 103.3 for unsprayed plots.

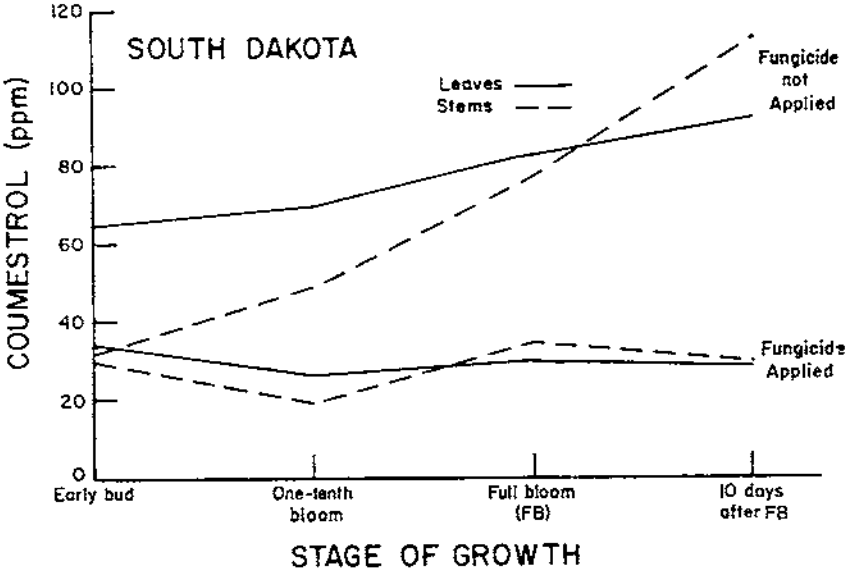

FIGURE 5.—Coumestrol content of leaves and stems of alfalfa cut at four stages of growth, with and without fungicide applications to control foliar diseases, Brookings, S. Dak., 1963.

Effect of Some Foliar Pathogens and Insect Pests on Coumestrol Content

This part of the study was concerned largely with greenhouse experiments conducted in 1963 on the effect of specific pathogens and insect pests on coumestrol content.

Materials and Methods

Several alfalfa disease specialists were requested to inoculate alfalfa with specific pathogens to obtain samples for comparing coumestrol contents of diseased and healthy forage. The pathogens, causal agents, and locations of the work were as follows: Spring blackstem (*Phoma herbarum* West. var. *medicaginis* Fckl.) at University Park, Pa.; common leaf spot (*Pseudopeziza medicaginis* (Lib.) Sacc.) at University Park and at St. Paul, Minn.; *Leptosphaerulina* leaf spot (*Leptosphaerulina briosiana* (Poll.) Graham & Luttrell) at University Park and St. Paul; *Stemphylium* leaf spot (*Stemphylium botryosum* Wallr.) at University Park; and alfalfa yellow mosaic virus at St. Paul. The first four are fungi. They cause leaf spotting and defoliation, and some of them also produce stem lesions. The virus disease symptom is leaf mottling. All these pathogens may cause yellowing of foliage and stunting.

Similarly, alfalfa was infested with the pea aphid (*Acyrthosiphon pisum* (Harris)) and the spotted alfalfa aphid (*Therioaphis maculata* (Buckton)) to determine the effect of insect infestation on coumestrol content.

Each of these studies was conducted in a greenhouse and a single field experiment followed. Alfalfa varieties and clones differentially affected by disease and insect damage in a field nursery at Beltsville, Md., were chosen for study. They obviously differed in quality of forage. Damage was caused by rust and the potato leafhopper (*Empoasca fabae* (Harris)). The alfalfa lines also differed in extent as to which saprophytic organisms had entered leaf tips suffering from potato leafhopper injury. Invasion caused surrounding tissue to turn black. Six varieties and clones representing a range in damage were scored and sampled for coumestrol determination on September 23, 1963. The last previous mowing of the plots was on August 11.

Plants growing under similar conditions except for the disease or insect differential imposed are referred to as a "set." Plants within a set were usually sampled in duplicate or triplicate. Samples were preserved in methanol and sent to Brookings, where coumestrol determinations were made as described on page 6.

Results

Effects of pathogens and insect pests on coumestrol content of forage are shown in tables 18–20. Comparisons should be limited to data within sets because environmental conditions and stages of growth differed between sets.

TABLE 18.—*Effect of 5 foliar pathogens on coumestrol content of alfalfa in greenhouse experiments, 1963*

Set No.	Location	Pathogen	Alfalfa variety or clone	Stage of growth	Part of foliage analyzed	Coumestrol content	
						Inoculated with pathogen	Healthy check
						P.p.m.	*P.p.m.*
1	University Park, Pa.	*Phoma herbarum* var. *medicaginis*	Buffalo	Prebud	Whole	219.1	0
						181.9	1.4
2	do	*Pseudopeziza medicaginis*	do	¼ bloom	do	59.5	0
						74.2	0
3	do	*Pseudopeziza medicaginis*	Vernal	Prebud	do	33.2	0
						48.4	0
4	St. Paul, Minn.	*Pseudopeziza medicaginis*	Clone R–5	Full bloom	do	9.1	2.0
5	University Park, Pa.	*Leptosphaerulina briosiana*	Vernal	Prebud	do	0	0
						0	0
6	St. Paul, Minn.	*Leptosphaerulina briosiana*	Clone R–5	Late bud	Whole	30.8	0
					Leaves	84.9	0
					Stems	0	0
7	University Park, Pa.	*Stemphylium botryosum*	Buffalo	Prebud	Whole	30.0	0
						41.5	0
8	St. Paul, Minn.	Yellow mosaic virus	Clone R–5	½ bloom	do	0	0
						0	0
9	do	do	Ranger	1/10 bloom	Whole	29.5	19.0
					Leaves	32.7	----
					Stems	18.8	----

TABLE 19.—*Effect of pea aphid and spotted alfalfa aphid infestations on coumestrol content of alfalfa clone 50–1266, Manhattan, Kans., in greenhouse experiments, 1965*

Set No.	Insect pest	Stage of alfalfa growth	Coumestrol content	
			Infested	Not infested
			P.p.m.	*P.p.m.*
1	Pea aphid	Bud	125.8	78.8
			199.2	90.2
2	Spotted alfalfa aphid	1/10 bloom	101.9	32.4
			201.0	53.8

TABLE 20.—*Coumestrol content of field-grown alfalfa varieties and clones, representing wide range of disease and insect damage, Beltsville, Md.*

Clone or variety	Rust [1]	Saprophyte [2]	Leafhopper yellowing [1]	Coumestrol content
	Score	*Score*	*Score*	*P.p.m.*
Clone BW19	2	5	5	119.2
Lahontan	5	3	4	91.7
Atlantic	4	3	4	78.5
Clone BW171	2	0	1	49.9
Williamsburg	3	2	3	43.7
Vernal	1	1	2	26.5

[1] 0=no damage; 5=severe damage.
[2] 0=no visible evidence of saprophyte; 5=extensive blackening of leaf tips, indicating invasion of saprophytes into areas damaged by potato leafhopper.

Fungi.—Infection by each of four fungi generally caused a marked increase in coumestrol content (table 18). The only inconsistency was found in samples inoculated with *L. briosiana.* Samples from University Park were from plants only 3 to 6 inches high in the vegetative stage. The absence of coumestrol in these samples may have been due to the early stage of growth, because infected samples from a later stage of growth at St. Paul contained coumestrol.

Highest coumestrol content was found in samples infected with *P. herbarum* var. *medicaginis* from University Park; infected samples contained an average of 200.5 as compared with 0.7 p.p.m. for the healthy check. Coumestrol was detected in only 2 of 17 healthy samples (all pathogens considered).

Virus.—Coumestrol was detected neither in virus-infected nor in healthy plants sampled in the first set (table 18). However, in the second set obtained from the same source several months later, coumestrol was found in the healthy as well as the infected plants, but a greater amount was found in the infected. Absence of virus in healthy plants was not verified.

Aphids.—Infestation by the pea aphid and the spotted alfalfa aphid increased the coumestrol content (table 19). However, noninfested plants contained a sizable quantity of coumestrol, which was unexpected. Infested and noninfested plants were judged to be free of fungal pathogens, but they had been infested with aphids 40 days

previously for another study. Although the old infested growth had been removed, it is possible that coumestrol could have been translocated into regrowth.

Miscellany.—The six varieties and clones sampled in the field planting represented a range of foliar damage in the planting. When they were arranged according to coumestrol content, they were in approximate order of average score for rust, invasion by saprophytes, and leafhopper yellowing (table 20). This ranking also appeared to be approximate for forage quality; the top-ranking entry was the poorest. The principal exception was clone BW171, which ranked one or two places higher than expected. Coumestrol contents of the three highest and three lowest entries averaged 96.5 and 40.0 p.p.m., respectively.

Effect of Aerobic and Anaerobic Storage Conditions on Coumestrol Content

Since infecting alfalfa plants in the field or greenhouse with foliar pathogens increased coumestrol content of the foliage, a question of primary interest was whether coumestrol content of these infected plants would be increased after harvesting and storage.

Materials and Methods

An experiment was made to determine the effect of aerobic and anaerobic storage on the coumestrol content of freshly cut alfalfa forage. Lightly and heavily diseased forage was stored at 38° and 85° F. Humid conditions were used throughout. Aerobic conditions simulated unfavorable field-curing conditions, which would normally result in development of molds and decomposition. Anaerobic conditions might approximate those generally associated with silage production.

Two 3-year-old fields at Brookings, S. Dak., of Ranger alfalfa that differed in severity of foliar disease infection were harvested in late August 1963 to obtain samples for storage. Alfalfa in the first field, cut last on July 15, 1963, was in about the ⅔-bloom stage, 23 inches tall, and heavily infected by foliar pathogens caused predominantly by *Pseudopeziza medicaginis* and *Cercospora zebrina*. Alfalfa in the second field, cut last on July 29, 1963, was in the late-bud stage, 20 inches tall, and nearly free of foliar diseases. Forage in each field was cut at mower height and chopped into 2-inch lengths with a papercutter.

Forage was obtained from the first field on August 27 for aerobic storage. About 125 grams of chopped forage were placed in each of 24 perforated cellophane bags. Half the bags were stored at 85° F. and the remainder at 38°. On August 29, 24 more samples were obtained from the same field and placed in 1-quart mason jars for anaerobic storage. To attain the latter, jars were evacuated with a vacuum pump, filled with nitrogen, and reevacuated. Similarly, samples from the second field were harvested and stored on corresponding dates. Thus, eight combinations of treatments were obtained from storing lightly and heavily infected samples under aerobic and anaerobic conditions at two temperatures. Also, 12 samples were stored for each of the eight treatment combinations to determine the effect of time in storage on coumestrol content.

Coumestrol determinations were made daily during the first 4 days of storage and on alternate days during the remaining 14 days of the experiment. Approximately 90 grams (wet weight) of each sample were placed in an 8-ounce, screw-cap jar containing 140 ml. of 100-percent methanol for extraction of coumestrol. Coumestrol content was determined as given on page 6.

Results

Coumestrol content of freshly cut alfalfa from the heavily infected field ranged from 70 to 83 p.p.m., whereas fresh samples from the lightly infected field ranged from 16.5 to 17.5 p.p.m. (fig. 6).

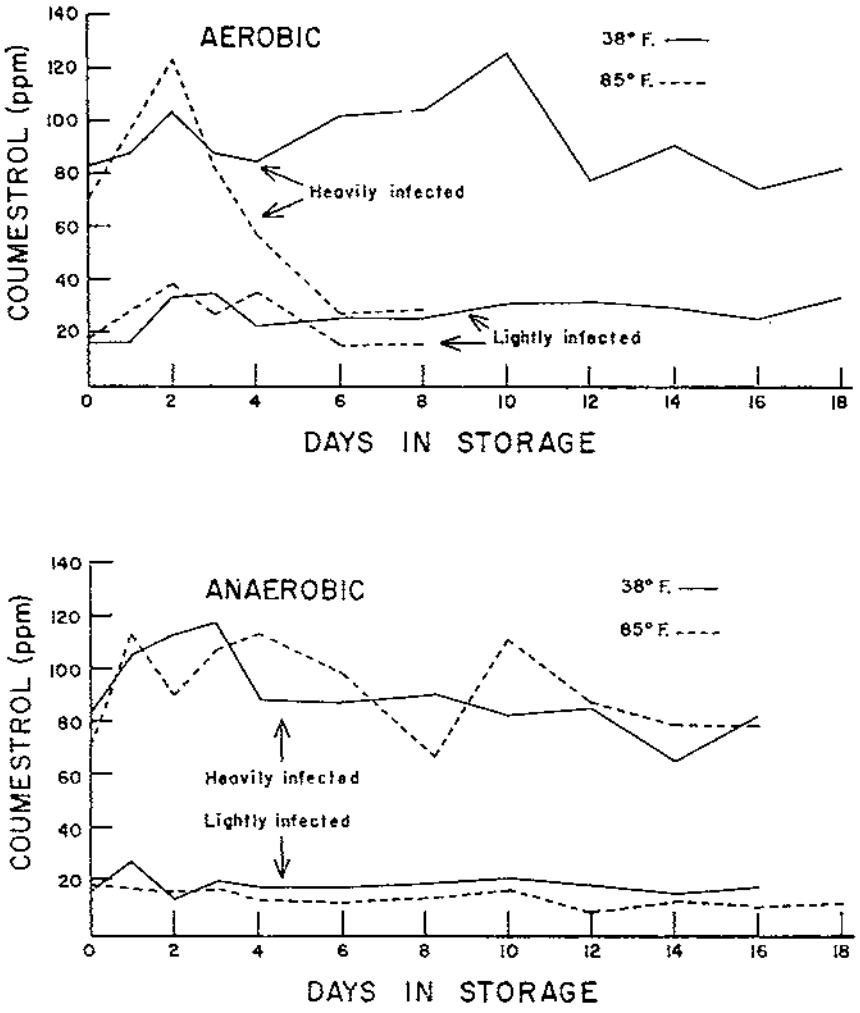

FIGURE 6.—Coumestrol content of alfalfa forage, lightly and heavily infected with foliar diseases, and stored at 38° and 85° F., Brookings, S. Dak., 1963. Above, aerobic storage conditions; below, anaerobic.

Coumestrol content of the heavily infected forage increased during the first 2 days of storage, but appeared to level off slightly above the original amount except in samples stored under aerobic conditions at 85° F. Coumestrol content of the latter declined rapidly after the second day and reached 26 p.p.m. on the sixth day. Determinations for this treatment combination were discontinued after the eighth day because samples had begun to decompose. No consistent or marked change occurred in coumestrol content of the lightly infected hay during storage except under aerobic conditions at 85°, where it dropped slightly after the fourth day. Chemical analysis of the latter was discontinued after the eighth day because of decomposition. Except for the decline occurring under aerobic conditions at 85°, changes in coumestrol content after harvesting were much less important than those resulting when living plants were infected by foliar pathogens.

DISCUSSION

Nature of Variation in Coumestrol Content at One-tenth-Bloom Stage

Nearly all variation in coumestrol content at the $\frac{1}{10}$-bloom stage was nongenetic. The variety component was only 0.8 percent of the plot component. Average effect of cuttings in the 2 years sampled on coumestrol content was small and estimated to be zero, but cuttings differed greatly at individual locations.

If either a high or low coumestrol level were desired, selection of location for growing the alfalfa should receive consideration, other economic factors being equal. Over the 2-year period average coumestrol contents for alfalfa from California and Utah were 10.4 and 12.6 p.p.m., respectively, as compared with 49.2 (Nebraska), 52.0 (North Carolina), 71.4 (Kansas), 87.7 (Pennsylvania), and 125.4 p.p.m. (Iowa) for the other locations. In California and Utah, coumestrol content was uniformly low, but at other locations cuttings varied greatly within years. Cuttings in Iowa, for example, ranged from 16.6 to 217.6 p.p.m. during the 2-year period. For commercial production of alfalfa with specified coumestrol content, chemical determination of each crop and production field would probably be necessary.

The pattern of variation for coumestrol content was very different from that for saponin content (*21*), in which effect of varieties was much larger and environmental effects were smaller.

Associations Between Coumestrol Content and Other Variables

Soil moisture, sunshine, and temperatures shortly before harvest were not associated with coumestrol content. These data were not adequate, however, to critically determine specific effects of environmental components on coumestrol content.

Coumestrol contents of all samples from the shading experiment in Utah were low and similar in magnitude to those of other samples harvested in Utah and California. Thus, the shading experiment did not support a hypothesis that low coumestrol content of Utah and California samples might be associated with high light intensity.

In interpreting the associations between coumestrol and the other characters at the variety level, it is necessary to consider characteristics of the varieties. Du Puits is slightly taller and earlier blooming than the other varieties. Du Puits and Vernal are the most resistant, and Lahontan the least resistant to foliar diseases. Buffalo and Ranger are intermediate, but Buffalo (with the exception of yellow leaf blotch) is slightly more resistant than Ranger. Defoliation of varieties in these studies was expected to be proportional to susceptibility, and this was found.

Also, locations have certain characteristics with regard to incidence of foliar disease. Disease incidence is usually lower in Utah and California than in other States in the study. Foliar diseases occur frequently in North Carolina and Pennsylvania. Kansas, Nebraska, and Iowa tend to be intermediate, but are more like the humid Eastern than arid Western States. Severe infection and defoliation occur frequently in the Central States when environmental conditions are favorable. In each of the seven States, incidence of foliar diseases in any one cutting appears to depend primarily on suitable moisture and temperature.

Because varieties ranked similarly for defoliation score and coumestrol content, the two characters were highly correlated at the variety level. The two characters were also significantly correlated at the location level and for five other sources of variation. It is significant that the association of coumestrol content with defoliation score was stronger than that between coumestrol and any other character.

After defoliation score, coumestrol content was correlated strongest with height when the comparison was based on proportion of significant correlations. However, signs of significant correlation coefficients were positive and negative, indicating an inconsistent relationship between height and coumestrol content. Some significant negative correlations were found between nitrogen-free extract and coumestrol content in individual and combined analysis of locations. However, this association was weak. Associations between coumestrol content and other characters were insignificant.

Controlling Coumestrol Content by Adjusting Stage of Harvest

Changes in contents of protein and other chemical constituents as related to stage of maturity were in agreement with previous work. Because coumestrol content generally increased with successive stages of growth, the following question arises: Would altering the time-of-cutting recommendations be warranted in order to raise or lower coumestrol content?

Previous work demonstrated that cutting at the $\frac{1}{10}$-bloom stage was about optimum when yield of dry matter, quality, and stand persistence were considered. Feasibility of cutting alfalfa at earlier or later stages of growth to change coumestrol content appears to depend on the value of altered coumestrol in the feed in relation to changes in yield, persistence, and quality.

On the basis of available information, cutting earlier than the $\frac{1}{10}$-bloom stage would generally result in lower dry-matter yields and lower fiber and coumestrol contents, but also would result in higher contents of protein and other constituents indicative of high quality.

The length of time that the stand would be expected to remain productive would be reduced with consistent earlier harvesting.

Cutting later than the 1/10-bloom stage would generally produce higher tonnage of hay, greater incidence of foliar diseases, more leaf loss, higher contents of coumestrol and fiber, greater persistence, and lower contents of protein and carotene. Therefore, a sizable value must be placed on altered coumestrol content to justify delaying the stage of harvest. Shifting the harvest stage to alter coumestrol content did not appear practical.

Changing Coumestrol Content by Breeding

Because more than 99 percent of the plot variation was estimated to be environmental, breeding for high or low coumestrol content per se did not appear promising. It should be noted, however, that the extent to which coumestrol content could be changed by selection depends also on variation among individual plants, which was not measured in this study. Wide variation in estrogenic activity was reported among clones harvested in the bud stage (*43*). Nevertheless, information obtained in the present investigation was not encouraging from the standpoint of breeding for high or low coumestrol content in alfalfa. There is one possible exception. Breeding varieties resistant to foliar diseases might be practical if low coumestrol were the objective. However, information is needed on coumestrol synthesis in resistant plants before knowing whether such an approach would be successful.

Effect of Foliar Diseases on Coumestrol Content

These investigations confirmed findings (*27*) that infections with foliar pathogens usually resulted in accumulation of coumestrol in the forage.

There were three kinds of direct evidence. First, in controlled inoculation experiments, infection with each of four fungi and one virus generally resulted in a marked increase in coumestrol content. The coumestrol content of all inoculated samples ranged from 0 to 219.1 and averaged 47.0 p.p.m. The healthy checks ranged from 0 to 19.0 and averaged 1.4 p.p.m. Coumestrol was detected in only 3 of 20 healthy samples.

Second, coumestrol content of forage increased with successive stages of growth in unsprayed alfalfa in a field experiment conducted at Brookings, S. Dak. Coumestrol content of plots sprayed with fungicide was relatively low and constant over stages of growth. On the last sampling date, average coumestrol content of stems and leaves from unsprayed plots was 103.3 p.p.m. as compared with 28.6 p.p.m. for sprayed plots. Defoliation and disease symptoms were much more severe in unsprayed than sprayed plots. It was concluded that increase in coumestrol content with advancing maturity in untreated plots was due primarily to increased infection by foliar pathogens, because coumestrol content was nearly constant in plots receiving fungicidal applications. Although there are no known direct effects of Dithane-45 on coumestrol synthesis, an alternate explanation attributing the control to direct effects of Dithane-45 on coumestrol synthesis could not be ruled out.

Third, in a storage study, changes in coumestrol content that occurred after harvesting were less important than those resulting when living plants were infected with foliar pathogens. An exception was storage under conditions favorable for decomposition, when coumestrol content declined. Coumestrol content of freshly cut alfalfa from fields heavily and lightly infected by foliar pathogens averaged 76 and 17 p.p.m., respectively. Coumestrol content of heavily infected forage increased some during the first 2 days of storage, but this appeared to stabilize at a level slightly above that at the time of harvest. Coumestrol contents of samples stored under aerobic conditions at 85° F. declined sharply after 2 days in storage, apparently because they began to decompose.

The following indirect evidence was also obtained linking foliar diseases to accumulation of coumestrol in forage:

(1) Ninety-nine percent of the coumestrol variation was nongenetic among samples of five varieties grown in seven States and harvested three times annually at the $\frac{1}{10}$-bloom stage in each of 2 years.

(2) Regression analysis indicated a closer relationship between coumestrol content and defoliation than between coumestrol content and any other character studied. Furthermore, ranking of varieties for defoliation was the same as a ranking expected for average resistance to foliar diseases. Lahontan, for example, had the greatest leaf loss, highest coumestrol content, and from past records was the most susceptible to foliar diseases.

(3) California and Utah, where alfalfa had a low coumestrol content, normally have a low incidence of foliar diseases.

(4) Varieties averaged over locations, years, and cuttings ranked the same for defoliation and coumestrol content. (It was assumed that foliar diseases were the principal cause of defoliation.)

(5) Coumestrol content generally increased with successive stages of growth in experiments at Lincoln, Nebr., University Park, Pa., and Davis, Calif. It was assumed that the increase resulted from a greater incidence of disease with advancing maturity, which is a common occurrence.

There was strong implication in these investigations that foliar pathogens and insect pests were the principal agents associated with accumulation of coumestrol in alfalfa forage. A literature review by Farkas and Kiraly (*17*) indicated that accumulation of phenolic substances in the plant commonly follows infection by pathogens. According to these workers, this accumulation in most attacked plants is due to the responsive reaction of the plant tissues. The mechanism of aromatic biosynthesis in diseased tissues is still unknown.

SUMMARY

These studies were undertaken to determine the effects of location, variety, cutting, year, stage of growth, light intensity, and plant pathogens on coumestrol content. Some information was also obtained on the effect of insect pests.

Variation at $\frac{1}{10}$-Bloom Stage.—The varieties Buffalo, Du Puits, Lahontan, Ranger, and Vernal were seeded in replicated plots at Davis, Calif., Ames, Iowa, Manhattan, Kans., Lincoln, Nebr., Raleigh, N.C., University Park, Pa., and Logan, Utah. Plots were harvested in 1960 and 1961. Data from the five varieties, three cuttings each

year, seven locations, and 2 years constituted the "core" of the experiment on which combined statistical analyses were conducted; additional cuttings were studied at some locations.

More than 99 percent of the variation was nongenetic as determined by variance components. The components for locations, cuttings×years, and cuttings×years×locations were similar in magnitude and accounted for about 72 percent of the variation. Varieties accounted for only 0.8 percent.

Differences among varieties for coumestrol content were small but significant. Lahontan had the highest coumestrol content; Du Puits and Vernal had the lowest. Cutting averages did not differ, but cuttings within locations varied greatly.

Coumestrol contents were lowest for forage from California and Utah, averaging 10.4 and 12.6 p.p.m., respectively. Highest values were obtained from Iowa, which averaged 125.4 p.p.m. Coumestrol contents of forage from other locations were intermediate—Nebraska 49.2 p.p.m., North Carolina 52.0, Kansas 71.4, and Pennsylvania 87.7. Averages for 1960 and 1961 were 59.8 and 57.0 p.p.m., respectively.

Coumestrol content was positively correlated with defoliation score, but not correlated with other characteristics, which included chemical constituents in the plant such as protein and fiber.

Variation as Affected by Growth Stage.—In a separate experiment, Buffalo and Ranger were established in replicated plots at Lincoln, Nebr., Davis, Calif., and University Park, Pa., and were harvested at six stages, ranging from 10 inches high to 25 days after full bloom.

Coumestrol content usually increased with successive stages of growth and reached a maximum 25 days after full bloom. Coumestrol content of cuttings within years and locations (averaged over stages) ranged from 0.5 p.p.m. for the second cutting of 1960 in California to 156.4 p.p.m. for the first cutting of 1961 in Pennsylvania. Highest content within stages was 429.2 p.p.m. for Ranger (averaged over replications) 25 days after full bloom in the second cutting of 1960 in Nebraska.

Over stages of growth, coumestrol was negatively correlated with protein content and positively correlated with crude fiber content. Changing harvest stage to alter coumestrol content did not appear practical.

Effect of Light Intensity.—When light intensity was reduced 72 percent with shade cloth in a field experiment at Logan, Utah, coumestrol content ranged from 3.0 to 12.0 p.p.m. and averaged 7.4 and 5.7 p.p.m., respectively, for shaded and unshaded plots. The effects of stage of growth, variety, and shading on coumestrol content were not significant.

Effect of Foliar Diseases.—In each of four experiments, accumulation of coumestrol usually occurred in forage after infection by foliar pathogens and thus confirmed previous results.

In one experiment, coumestrol increased with successive stages of growth when alfalfa was left unsprayed in a field experiment at Brookings, S. Dak. Spraying with a fungicide to reduce foliar diseases resulted in relatively low and constant levels of coumestrol. On the last sampling date, average content of leaves and stems from unsprayed plots was 103.3 as compared with 28.6 p.p.m. for sprayed plots.

In another experiment, infection by each of four fungi and one virus generally resulted in marked increases of coumestrol content. The

content of inoculated samples ranged from 0 to 219.1 and averaged 47.0 p.p.m. The healthy checks ranged from 0 to 19.0 and averaged 1.4 p.p.m. Coumestrol was detected in only 3 of 20 healthy samples.

Lightly and heavily infected alfalfa forage was stored under aerobic and anaerobic conditions at 38° and 85° F. Changes in coumestrol content occurring during an 18-day period after harvesting were less important than those resulting when living plants were infected with foliar pathogens. An exception was under conditions favorable for decomposition, when coumestrol content declined rapidly. Contents of the lightly and heavily infected forage at harvest were 17 and 76 p.p.m., respectively.

Six varieties and clones representing a range of foliar damage in a field planting at Beltsville, Md., arranged according to coumestrol content (high to low), were in approximate order of average score for rust, leaf tip invasion by saprophytes, and leafhopper yellowing (highest score = most damage).

The following indirect evidence from other phases of this investigation also linked foliar disease infection with accumulation of coumestrol in forage: (1) More than 99 percent of variation at the 1/10-bloom stage was nongenetic. (2) Coumestrol content was correlated with defoliation score. (3) California and Utah, where alfalfa had a low coumestrol content, normally have a low incidence of foliar diseases. (4) Coumestrol content generally increased with stages of growth, which is consistent with a common observation that disease incidence increases with advancing maturity.

Effect of Aphids.—Preliminary data indicated that infestation with pea aphids and spotted alfalfa aphids increased coumestrol content. However, noninfested check plants contained a sizable quantity of coumestrol, which was unexpected.

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APPENDIX

TABLE A1.—*Average coumestrol content, height, defoliation and maturity scores, and other chemical constituents for varieties and cuttings of alfalfa, Davis, Calif.*

Variety	Coumestrol		Height		Defoliation		Maturity		Protein	Crude fiber	Ash	Fat	N-free extract
	1960	1961	1960	1961	1960	1961	1960	1961	1960	1960	1960	1960	1960
	P.p.m.		*Inches*		*Score* [1]		*Score* [1]		*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1ST CUTTING													
Buffalo	30.5	5.0	23.5	28.0	0	0	2.0	2.0	20.0	28.0	10.3	2.6	39.1
Du Puits	10.8	1.0	28.5	32.0	0	.5	2.0	2.5	20.8	25.7	10.9	3.3	39.3
Lahontan	51.8	13.3	26.8	30.5	0	0	2.0	2.0	20.1	25.9	9.8	2.9	41.4
Ranger	35.5	4.0	25.0	27.0	0	0	2.0	2.0	21.2	25.3	10.0	3.0	39.6
Vernal	26.8	5.3	24.0	30.5	0	.3	2.0	2.0	21.7	24.5	10.2	3.2	40.4
Average	31.1	5.7	25.6	29.6	0	.2	2.0	2.1	20.8	25.9	10.4	3.0	40.0
2D CUTTING													
Buffalo	15.5	5.8	26.5	30.3	1.8	2.0	2.0	2.0	22.7	27.3	11.0	2.5	36.5
Du Puits	16.0	2.8	29.0	31.8	1.0	1.0	2.0	2.0	20.1	29.2	10.5	2.7	37.5
Lahontan	25.5	2.8	26.8	31.8	1.0	1.0	2.0	2.0	21.2	27.7	10.4	2.6	38.1
Ranger	13.3	3.3	35.0	30.3	1.3	2.0	2.0	2.0	23.5	26.3	10.8	2.6	36.8
Vernal	12.8	1.8	23.8	30.3	1.0	1.0	2.0	2.0	23.0	26.9	10.6	2.6	36.8
Average	16.6	3.3	28.2	30.9	1.2	1.4	2.0	2.0	22.1	27.5	10.7	2.6	37.1
3D CUTTING													
Buffalo	0	7.5	28.0	29.3	.5	1.0	3.0	2.0	19.9	27.1	10.2	2.8	40.1
Du Puits	0	2.0	32.8	28.5	.5	1.0	3.0	2.0	18.8	28.8	10.0	2.7	39.8
Lahontan	.8	5.0	30.3	31.0	1.3	1.0	3.0	2.0	20.9	25.1	10.6	2.8	40.5
Ranger	0	9.0	28.8	28.5	.5	1.0	3.0	2.0	21.1	24.8	10.4	2.9	40.9
Vernal	0	6.5	26.8	29.5	.3	1.0	3.0	2.0	20.7	28.7	10.3	2.9	37.3
Average	.2	6.0	29.3	29.4	.6	1.0	3.0	2.0	20.3	26.9	10.3	2.8	39.7

4TH CUTTING													
Buffalo	4.0	14.0	37.5	23.5	0	1.0	2.0	2.0	20.3	25.3	11.4	2.4	40.6
Du Puits	0	10.8	41.0	27.5	1.0	1.0	2.0	2.0	18.8	29.1	10.7	2.8	38.6
Lahontan	0	9.8	26.3	26.5	2.0	1.0	2.0	2.0	20.0	28.2	11.4	3.0	37.4
Ranger	0	13.8	35.3	22.3	.5	1.0	2.0	2.0	19.3	24.1	11.3	2.5	42.8
Vernal	2.8	16.0	35.3	24.0	1.0	1.5	2.0	2.0	18.8	26.3	11.3	2.5	41.1
Average	1.4	12.9	35.1	24.8	.9	1.1	2.0	2.0	19.4	26.6	11.2	2.6	40.1
AVERAGE OF 4 CUTTINGS													
Buffalo	12.5	8.1	28.9	27.8	.6	1.0	2.2	2.0	20.7	26.9	10.7	2.6	39.1
Du Puits	6.7	4.2	32.8	30.0	.6	.9	2.2	2.1	19.6	28.2	10.5	2.9	38.8
Lahontan	19.5	7.7	27.6	30.0	1.1	.8	2.2	2.0	20.6	26.7	10.6	2.8	39.4
Ranger	12.2	7.5	31.0	27.0	.6	1.0	2.2	2.0	21.3	25.1	10.8	2.8	40.0
Vernal	10.6	7.4	27.5	28.6	.6	1.0	2.2	2.0	21.0	26.6	10.6	2.8	38.9
Average	12.3	7.0	29.6	28.7	.7	.9	2.2	2.0	20.6	26.7	10.6	2.8	39.2
5TH CUTTING													
Buffalo	3.5	-------	28.0	-------	1.0	-------	2.0	-------	21.0	22.7	12.3	3.2	40.8
Du Puits	6.8	-------	29.5	-------	1.0	-------	2.0	-------	19.3	24.9	11.4	3.0	41.4
Lahontan	11.8	-------	27.5	-------	1.0	-------	2.0	-------	20.6	21.6	11.6	3.1	43.2
Ranger	6.8	-------	23.3	-------	1.0	-------	2.0	-------	23.7	24.1	12.0	3.4	36.8
Vernal	38.5	-------	24.8	-------	1.8	-------	2.0	-------	21.0	20.3	12.1	3.4	43.2
Average	13.5	-------	26.6	-------	1.2	-------	2.0	-------	21.1	22.7	11.8	3.2	41.1

[1] From 0 to 7, representing 0- to 70-percent or greater defoliation, respectively.
[2] 1=late-bud stage, 2=1/10-bloom stage, 3=½-bloom stage, and 4=full bloom.

TABLE A2.—*Average coumestrol content, height, defoliation and maturity scores, and other chemical constituents for varieties and cuttings of alfalfa, Ames, Iowa*

Variety	Coumestrol		Height		Defoliation		Maturity		Protein	Crude fiber	Ash	Fat	N-free extract
	1960	1961	1960	1961	1960	1961	1960	1961	1960	1960	1960	1960	1960
	P.p.m.		*Inches*		*Score* [1]		*Score* [1]		*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1ST CUTTING													
Buffalo	126.8	237.0	-------	16.5	-------	-------	3.0	2.0	19.0	31.6	7.9	3.0	38.6
Du Puits	103.0	107.0	-------	23.8	-------	-------	4.0	3.0	18.6	31.4	8.1	3.1	38.8
Lahontan	98.8	257.0	-------	14.5	-------	-------	3.0	2.0	19.1	31.4	8.3	3.0	38.2
Ranger	97.8	136.0	-------	15.8	-------	-------	3.0	2.0	18.1	29.5	7.6	2.9	41.8
Vernal	65.8	123.8	-------	15.8	-------	-------	3.0	2.0	17.8	32.4	7.5	2.7	39.6
Average	98.4	172.2	-------	17.3	-------	-------	3.2	2.2	18.5	31.2	7.9	2.9	39.4
2D CUTTING													
Buffalo	109.3	15.3	-------	15.5	-------	-------	2.0	2.0	20.0	23.4	7.7	3.1	45.8
Du Puits	116.0	19.5	-------	18.8	-------	-------	2.0	2.0	18.5	25.3	7.2	2.9	46.2
Lahontan	140.5	16.5	-------	14.5	-------	-------	2.0	2.0	19.5	28.1	7.2	2.7	42.4
Ranger	137.8	18.5	-------	15.0	-------	-------	2.0	2.0	18.7	27.0	7.4	2.8	44.0
Vernal	117.5	13.3	-------	14.8	-------	-------	2.0	2.0	20.2	28.7	7.1	3.1	40.8
Average	124.2	16.6	-------	15.7	-------	-------	2.0	2.0	19.4	26.5	7.3	2.9	43.9

	3D CUTTING												
Buffalo	169.8	115.0	------	15.5	------	------	2.0	2.0	22.9	26.4	7.8	3.1	39.9
Du Puits	146.0	150.8	------	16.3	------	------	2.0	2.0	22.3	25.1	7.7	3.0	41.9
Lahontan	308.3	111.5	------	14.5	------	------	2.0	2.0	21.1	29.6	7.5	2.7	39.2
Ranger	215.0	129.3	------	13.8	------	------	2.0	2.0	23.1	26.4	8.1	3.6	38.9
Vernal	249.0	112.5	------	13.8	------	------	2.0	2.0	23.2	23.9	7.6	3.7	41.7
Average	217.6	123.8	------	14.8	------	------	2.0	2.0	22.5	26.3	7.7	3.2	40.3
	AVERAGE OF 3 CUTTINGS												
Buffalo	135.3	122.4	------	15.8	------	------	2.3	2.0	20.6	27.1	7.8	3.1	41.4
Du Puits	121.7	92.4	------	19.6	------	------	2.7	2.3	19.8	27.3	7.7	3.0	42.3
Lahontan	182.5	128.3	------	14.5	------	------	2.3	2.0	19.9	29.7	7.7	2.8	39.9
Ranger	150.2	94.6	------	14.8	------	------	2.3	2.0	20.0	27.6	7.7	3.1	41.5
Vernal	144.1	83.2	------	14.8	------	------	2.3	2.0	20.4	28.3	7.4	3.2	40.7
Average	146.7	104.2	------	15.9	------	------	2.4	2.1	20.1	28.0	7.6	3.0	41.2

[1] See table A1, footnote 2.

TABLE A3.—*Average coumestrol content, height, defoliation and maturity scores, and other chemical constituents for varieties and cuttings of alfalfa, Manhattan, Kans.*

Variety	Coumestrol		Height		Defoliation		Maturity		Protein	Crude fiber	Ash	Fat	N-free extract
	1960	1961	1960	1961	1960	1961	1960	1961	1960	1960	1960	1960	1960
	P.p.m.		*Inches*		*Score* [1]		*Score* [2]		*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1ST CUTTING													
Buffalo	10. 5	261. 0	29. 5	24. 3	0	3. 5	2. 0	2. 0	19. 5	24. 8	11. 8	3. 3	40. 6
Du Puits	8. 3	186. 5	29. 8	26. 3	0	3. 0	2. 0	2. 0	19. 8	21. 6	12. 2	3. 3	43. 2
Lahontan	9. 5	284. 0	28. 8	23. 3	0	4. 0	2. 0	2. 0	19. 1	28. 1	11. 5	3. 5	37. 8
Ranger	12. 3	295. 8	29. 5	23. 3	0	4. 0	2. 0	2. 0	19. 7	25. 5	11. 7	3. 4	39. 6
Vernal	11. 0	208. 3	29. 3	23. 0	0	4. 0	2. 0	2. 0	19. 3	25. 5	11. 8	3. 8	39. 6
Average	10. 3	247. 1	29. 4	24. 0	0	3. 7	2. 0	2. 0	19. 5	25. 1	11. 8	3. 5	40. 1
2D CUTTING													
Buffalo	33. 5	13. 8	20. 3	24. 8	2. 8	0	2. 0	2. 0	18. 9	27. 4	12. 1	3. 0	38. 6
Du Puits	64. 8	16. 0	23. 3	27. 0	2. 8	0	2. 0	2. 0	21. 9	27. 7	11. 1	3. 6	35. 7
Lahontan	51. 0	16. 5	21. 8	24. 8	3. 8	0	2. 0	2. 0	18. 8	26. 6	11. 2	3. 1	40. 3
Ranger	26. 8	16. 8	21. 5	23. 3	2. 8	0	2. 0	2. 0	18. 5	26. 7	11. 4	3. 1	40. 2
Vernal	27. 3	10. 5	20. 3	23. 8	2. 8	0	2. 0	2. 0	18. 1	29. 6	10. 6	3. 4	38. 2
Average	40. 7	14. 7	21. 4	24. 7	3. 0	0	2. 0	2. 0	19. 2	27. 6	11. 3	3. 2	38. 6

	3D CUTTING												
Buffalo	57.8	46.0	24.0	26.3	4.0	2.8	2.0	2.0	19.3	26.3	10.5	3.1	40.8
Du Puits	71.8	44.5	22.8	27.0	3.8	3.0	2.0	2.0	19.8	27.5	10.0	3.2	39.5
Lahontan	97.8	53.0	21.0	26.0	4.8	4.0	2.0	2.0	20.0	29.6	10.1	3.3	36.9
Ranger	69.3	49.5	22.5	23.0	4.0	3.0	2.0	2.0	19.0	29.2	9.4	3.0	39.4
Vernal	54.8	34.8	22.0	23.0	4.0	2.8	2.0	2.0	19.7	28.4	9.6	3.2	39.0
Average	70.3	45.6	22.5	25.1	4.1	3.1	2.0	2.0	19.6	28.2	9.9	3.2	39.1
	4TH CUTTING												
Buffalo	36.3	29.0	19.8	26.3	3.3	3.0	2.0	2.0	22.2	26.8	11.1	3.3	36.6
Du Puits	44.8	27.0	19.0	26.5	3.3	3.0	2.0	2.0	23.0	24.0	10.8	3.6	38.5
Lahontan	45.0	45.0	19.3	26.0	4.0	4.0	2.0	2.0	22.1	27.4	10.5	4.2	35.9
Ranger	35.8	40.3	19.8	24.3	3.3	3.0	2.0	2.0	23.1	25.3	10.7	4.1	36.8
Vernal	44.5	36.3	21.3	22.5	3.0	3.0	2.0	2.0	22.8	25.5	10.8	3.6	37.3
Average	41.3	35.5	19.8	25.1	3.4	3.2	2.0	2.0	22.6	25.8	10.8	3.8	37.0
	AVERAGE OF 4 CUTTINGS												
Buffalo	34.5	87.4	23.4	25.4	2.5	2.3	2.0	2.0	20.0	26.3	11.4	3.2	39.2
Du Puits	47.4	68.5	23.7	26.7	2.4	2.3	2.0	2.0	21.1	25.2	11.0	3.4	39.2
Lahontan	50.8	99.6	22.7	25.0	3.1	3.0	2.0	2.0	20.0	27.9	10.8	3.5	37.7
Ranger	36.0	100.6	23.3	23.4	2.5	2.5	2.0	2.0	20.1	26.7	10.8	3.4	39.0
Vernal	34.4	72.4	23.2	23.1	2.4	2.4	2.0	2.0	20.0	27.2	10.7	3.5	38.5
Average	40.6	85.7	23.3	24.7	2.6	2.5	2.0	2.0	20.2	26.7	11.0	3.4	38.7

[1] See table A1, footnote 1.
[2] See table A1, footnote 2.

TABLE A4.—*Average coumestrol content, height, defoliation and maturity scores, and other chemical constituents for varieties and cuttings of alfalfa, Lincoln, Nebr.*

Variety	Coumestrol		Height		Defoliation		Maturity		Protein	Crude fiber	Ash	Fat	N-free extract
	1960	1961	1960	1961	1960	1961	1960	1961	1960	1960	1960	1960	1960
	P.p.m.		*Inches*		*Score* [1]		*Score* [2]		*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1ST CUTTING													
Buffalo	63.5	109.0	29.3	36.0	2.0	4.0	2.0	2.0	16.6	33.7	9.4	3.4	36.9
Du Puits	43.5	24.8	30.5	40.5	1.0	3.0	2.0	2.0	18.3	29.6	9.8	3.6	38.7
Lahontan	66.0	101.5	28.8	39.0	2.0	4.0	2.0	2.0	17.5	31.6	9.7	3.5	37.7
Ranger	72.8	118.5	27.8	34.5	2.0	4.0	2.0	2.0	16.8	32.0	9.4	3.4	38.4
Vernal	46.5	117.0	26.5	41.5	1.0	4.0	2.0	2.0	17.9	30.6	9.3	3.2	39.1
Average	58.5	94.2	28.6	38.3	1.6	3.8	2.0	2.0	17.4	31.5	9.5	3.4	38.2
2D CUTTING													
Buffalo	60.5	15.8	28.3	33.0	1.0	1.0	2.0	2.0	17.2	34.2	9.4	2.4	36.8
Du Puits	50.5	1.8	24.3	25.8	0	0	2.0	2.0	20.6	30.2	10.9	3.0	35.3
Lahontan	47.8	16.3	28.8	30.8	0	1.0	2.0	2.0	16.9	32.1	9.5	2.8	38.7
Ranger	81.8	18.0	26.8	31.0	1.0	1.0	2.0	2.0	17.0	34.0	9.4	2.3	37.2
Vernal	32.8	14.0	27.0	31.0	0	1.0	2.0	2.0	17.5	30.7	9.5	2.9	39.3
Average	54.7	13.2	27.0	30.3	.4	.8	2.0	2.0	17.9	32.2	9.8	2.7	37.5

	3D CUTTING												
Buffalo	52.3	18.8	25.3	29.3	3.0	2.0	2.0	2.0	17.4	32.7	9.2	2.5	38.2
Du Puits	35.5	15.8	23.8	27.5	1.0	2.0	2.0	2.0	20.9	29.2	9.3	3.7	36.9
Lahontan	89.5	21.8	23.3	27.3	2.8	2.0	2.0	2.0	19.6	31.4	9.4	3.1	36.5
Ranger	52.3	23.0	23.3	27.8	3.0	2.0	2.0	2.0	17.7	32.1	9.0	2.6	38.6
Vernal	47.8	17.3	23.5	26.0	3.0	2.0	2.0	2.0	18.5	32.0	9.2	3.0	37.4
Average	55.5	19.3	23.8	27.6	2.6	2.0	2.0	2.0	18.8	31.5	9.2	3.0	37.5
	AVERAGE OF 3 CUTTINGS												
Buffalo	58.8	47.8	27.6	32.8	2.0	2.3	2.0	2.0	17.1	33.5	9.3	2.8	37.3
Du Puits	43.2	14.1	26.2	31.3	.7	1.7	2.0	2.0	19.9	29.7	10.0	3.4	37.0
Lahontan	67.8	46.5	26.9	32.3	1.6	2.3	2.0	2.0	18.0	31.7	9.5	3.1	37.6
Ranger	68.9	53.2	25.9	31.1	2.0	2.3	2.0	2.0	17.2	32.7	9.3	2.8	38.1
Vernal	42.3	49.4	25.7	32.8	1.3	2.3	2.0	2.0	18.0	31.1	9.3	3.0	38.6
Average	56.2	42.2	26.5	32.1	1.5	2.2	2.0	2.0	18.0	31.7	9.5	3.0	37.7

[1] See table A1, footnote 1.
[2] See table A1, footnote 2.

TABLE A5.—*Average coumestrol content, height, defoliation and maturity scores, and other chemical constituents for varieties and cuttings of alfalfa, Raleigh, N.C.*

Variety	Coumestrol		Height		Defoliation		Maturity		Protein 1960	Crude fiber 1960	Ash 1960	Fat 1960	N-free extract 1960
	1960	1961	1960	1961	1960	1961	1960	1961					
	P.p.m.		*Inches*		*Score* [1]		*Score* [2]		*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1ST CUTTING													
Buffalo	13.0	56.0	23.8	16.5	1.5	2.0	2.0	2.3	22.2	22.0	10.1	3.3	42.4
Du Puits	12.0	47.8	25.0	20.0	1.8	1.8	2.0	2.0	21.6	23.9	10.2	3.3	41.0
Lahontan	16.5	60.0	19.0	14.3	1.8	1.3	2.0	2.0	22.6	22.9	9.8	3.7	41.0
Ranger	19.3	35.8	20.0	13.8	1.3	1.5	2.0	2.0	22.7	22.6	10.2	3.6	40.8
Vernal	13.0	42.0	21.0	16.0	1.0	1.5	2.0	2.0	22.2	22.2	10.1	3.6	41.9
Average	14.8	48.3	21.8	16.1	1.5	1.6	2.0	2.1	22.3	22.7	10.1	3.5	41.4
2D CUTTING													
Buffalo	61.8	40.5	15.3	27.8	1.3	3.0	2.0	2.0	27.5	19.0	9.9	4.1	39.4
Du Puits	76.0	19.8	16.3	33.8	.8	2.0	3.0	3.0	27.4	19.6	9.6	4.1	39.4
Lahontan	95.0	21.8	14.8	26.8	1.8	3.3	2.0	2.0	27.1	19.3	9.4	4.1	40.1
Ranger	91.0	20.3	13.5	26.5	.5	1.8	2.0	2.0	29.1	18.9	10.0	3.7	38.2
Vernal	93.0	23.5	14.8	26.5	0	2.3	2.0	2.0	29.3	18.7	9.9	3.9	38.3
Average	83.4	25.2	14.9	28.3	.9	2.5	2.2	2.2	28.1	19.1	9.8	4.0	39.1

	3D CUTTING												
Buffalo	99.3	46.0	19.0	28.8	1.5	1.5	2.3	2.0	24.3	23.9	9.6	3.5	38.6
Du Puits	133.5	58.5	18.3	33.3	1.5	1.5	3.3	3.0	24.5	21.8	9.6	3.5	40.7
Lahontan	99.3	37.8	17.3	30.5	2.3	1.3	3.3	2.3	23.2	24.5	9.0	3.3	40.0
Ranger	69.8	36.5	17.0	29.8	1.3	1.5	2.3	2.0	24.6	23.2	9.8	3.4	39.0
Vernal	83.0	39.8	16.8	31.3	1.8	1.0	2.0	2.0	25.7	22.2	9.7	3.5	38.9
Average	97.0	43.7	17.7	30.7	1.7	1.4	2.6	2.3	24.5	23.1	9.5	3.4	39.4
	4TH CUTTING												
Buffalo	42.8	43.5	22.3	28.3	2.0	3.3	2.0	2.0	19.5	28.3	9.5	2.8	40.0
Du Puits	40.3	40.8	24.3	26.0	2.5	3.8	2.0	2.8	19.7	28.5	9.8	2.7	39.3
Lahontan	61.5	65.0	22.0	25.8	4.0	6.8	2.0	2.5	20.0	28.7	9.0	2.6	39.7
Ranger	44.3	42.3	23.3	24.5	2.3	4.8	2.0	2.0	19.8	28.5	9.6	2.5	39.6
Vernal	46.5	38.3	22.5	24.3	2.3	4.3	2.0	2.0	20.9	27.0	10.0	3.2	38.9
Average	47.1	46.0	22.9	25.8	2.6	4.6	2.0	2.3	20.0	28.2	9.6	2.8	39.5
	AVERAGE OF 4 CUTTINGS												
Buffalo	54.2	46.5	20.1	25.3	1.6	2.4	2.1	2.1	23.4	23.3	9.8	3.4	40.1
Du Puits	65.4	41.7	20.9	28.3	1.6	2.3	2.6	2.7	23.3	23.4	9.8	3.4	40.1
Lahontan	68.1	46.1	18.3	24.3	2.4	3.1	2.3	2.2	23.2	23.8	9.3	3.4	40.2
Ranger	56.1	33.7	18.4	23.6	1.3	2.4	2.1	2.0	24.0	23.3	9.9	3.3	39.4
Vernal	58.9	35.9	18.8	24.5	1.3	2.3	2.0	2.0	24.5	22.5	9.9	3.6	39.5
Average	60.6	40.8	19.3	25.2	1.7	2.5	2.2	2.2	23.7	23.3	9.8	3.4	39.8

[1] See table A1, footnote 1.
[2] See table A1, footnote 2.

TABLE A6.—*Average coumestrol content, height, defoliation and maturity scores, and other chemical constituents for varieties and cuttings of alfalfa, University Park, Pa.*

Variety	Coumestrol		Height		Defoliation		Maturity		Protein	Crude fiber	Ash	Fat	N-free extract
	1960	1961	1960	1961	1960	1961	1960	1961	1960	1960	1960	1960	1960
	P.p.m.		*Inches*		*Score* [1]		*Score* [2]		*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1ST CUTTING													
Buffalo	160.3	134.0	21.3	28.8	2.0	4.8	1.0	2.0	22.3	29.2	10.6	3.1	34.8
Du Puits	74.5	149.3	25.3	31.3	2.0	4.5	1.0	2.0	21.3	29.3	10.5	2.9	36.0
Lahontan	62.8	146.0	21.5	30.8	2.0	5.5	1.0	2.0	20.9	29.3	10.2	2.9	36.6
Ranger	125.0	97.8	22.0	28.5	2.0	4.8	1.0	2.0	22.0	30.2	10.6	2.8	34.4
Vernal	69.8	136.8	22.5	33.5	2.0	4.8	1.0	2.0	21.0	31.4	10.3	2.9	34.5
Average	98.5	132.8	22.5	30.6	2.0	4.9	1.0	2.0	21.5	29.9	10.4	2.9	35.2
2D CUTTING													
Buffalo	72.0	28.3	17.5	15.8	2.0	1.3	2.0	2.0	18.3	30.1	8.9	3.0	39.7
Du Puits	70.0	48.3	19.8	16.8	2.0	1.0	2.0	2.0	17.9	31.6	8.6	3.1	38.8
Lahontan	89.3	41.8	17.8	13.3	3.0	.8	2.0	2.0	18.0	30.5	8.7	3.0	39.8
Ranger	88.0	49.0	17.3	13.0	2.0	.8	2.0	2.0	18.9	30.4	9.0	3.1	38.7
Vernal	75.5	42.3	16.8	16.3	1.0	1.0	2.0	2.0	19.1	30.6	8.7	3.4	38.2
Average	79.0	41.9	17.8	15.0	2.0	1.0	2.0	2.0	18.4	30.6	8.8	3.1	39.0

	3D CUTTING												
Buffalo	66.8	59.0	13.3	29.3	3.3	2.8	2.0	2.0	17.8	24.8	9.3	3.7	44.4
Du Puits	72.0	54.0	14.5	30.5	3.5	2.5	2.0	2.0	17.9	24.8	8.6	3.2	45.6
Lahontan	151.0	115.5	13.0	24.8	7.0	2.8	2.0	2.0	17.9	28.4	8.4	2.7	42.6
Ranger	104.0	86.8	12.0	24.3	5.0	2.8	2.0	2.0	18.1	25.6	8.6	3.1	44.6
Vernal	88.3	75.0	12.0	25.3	1.8	2.5	2.0	2.0	19.2	23.9	8.7	3.3	44.9
Average	96.4	78.1	13.0	26.8	4.1	2.7	2.0	2.0	18.2	25.5	8.7	3.2	44.4
	AVERAGE OF 3 CUTTINGS												
Buffalo	99.7	73.8	17.3	24.6	2.4	2.9	1.7	2.0	19.5	28.0	9.6	3.3	39.6
Du Puits	72.2	83.8	19.8	26.2	2.5	2.7	1.7	2.0	19.0	28.6	9.2	3.1	40.1
Lahontan	101.0	101.1	17.4	22.9	4.0	3.0	1.7	2.0	18.9	29.4	9.1	2.9	39.7
Ranger	105.7	77.8	17.1	21.9	3.0	2.8	1.7	2.0	19.7	28.7	9.4	3.0	39.2
Vernal	77.8	84.7	17.1	25.0	1.6	2.8	1.7	2.0	19.8	28.6	9.2	3.2	39.2
Average	91.3	84.2	17.8	24.1	2.7	2.8	1.7	2.0	19.4	28.7	9.3	3.1	39.6

[1] See table A1, footnote 1.
[2] See table A1, footnote 2.

TABLE A7.—*Average coumestrol content, height, defoliation and maturity scores, and other chemical constituents for varieties and cuttings of alfalfa, Logan, Utah*

Variety	Coumestrol		Height		Defoliation		Maturity		Protein	Crude fiber	Ash	Fat	N-free extract
	1960	1961	1960	1961	1960	1961	1960	1961	1960	1960	1960	1960	1960
	P.p.m.		*Inches*		*Score* [1]		*Score* [2]		*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1ST CUTTING													
Buffalo	3.8	47.0	15.8	26.8	1.0	3.0	1.3	2.0	18.9	20.7	12.2	3.7	44.6
Du Puits	3.5	55.8	15.5	26.8	1.0	1.3	2.0	2.0	19.6	17.5	11.0	3.8	48.2
Lahontan	5.0	40.5	16.0	24.5	1.0	3.5	1.3	1.0	18.9	16.7	11.8	4.1	48.4
Ranger	3.5	53.8	14.8	25.0	1.0	3.5	1.8	2.0	18.8	17.0	11.4	3.9	48.9
Vernal	2.8	49.3	15.5	23.8	1.0	2.5	1.8	2.3	18.4	17.3	11.8	4.0	48.5
Average	3.7	49.3	15.5	25.4	1.0	2.8	1.6	1.9	18.9	17.8	11.6	3.9	47.7
2D CUTTING													
Buffalo	0	3.5	16.3	28.0	1.0	.5	2.0	1.5	19.8	17.6	12.0	4.4	46.2
Du Puits	0	3.5	15.5	30.8	1.0	1.0	2.3	2.5	19.6	20.8	11.4	4.0	44.2
Lahontan	0	3.8	14.8	27.5	1.0	.8	1.5	1.5	20.5	17.3	11.9	4.5	45.9
Ranger	0	4.8	15.0	28.3	1.0	.5	1.8	1.3	20.5	18.1	11.6	3.9	46.0
Vernal	0	6.0	14.5	25.5	1.0	1.3	1.3	1.5	20.8	24.0	11.3	4.3	39.7
Average	0	4.3	15.2	28.0	1.0	.8	1.8	1.7	20.2	19.6	11.6	4.2	44.4

	3D CUTTING												
Buffalo	5.0	13.0	24.3	23.5	1.0	3.0	2.0	2.0	16.7	25.8	11.3	3.3	42.9
Du Puits	4.0	8.0	25.3	25.3	1.0	1.8	2.0	2.5	17.1	27.2	11.2	3.4	41.1
Lahontan	5.0	7.8	22.0	23.5	1.0	3.3	2.0	2.0	16.9	23.5	11.2	3.6	44.7
Ranger	7.0	11.3	22.8	21.8	1.0	3.3	2.0	2.0	18.3	22.5	11.3	3.6	44.3
Vernal	11.0	20.8	20.8	20.8	1.0	3.0	2.0	2.0	18.1	22.8	11.5	3.7	43.9
Average	6.4	12.2	23.0	23.0	1.0	2.9	2.0	2.1	17.4	24.4	11.3	3.5	43.4
	AVERAGE OF 3 CUTTINGS												
Buffalo	2.9	21.2	18.8	26.1	1.0	2.2	1.8	1.8	18.5	21.4	11.8	3.8	44.6
Du Puits	2.5	22.4	18.8	27.6	1.0	1.3	2.1	2.3	18.8	21.8	11.2	3.7	44.5
Lahontan	3.3	17.3	17.6	25.2	1.0	2.5	1.6	1.5	18.8	19.2	11.6	4.1	46.3
Ranger	3.5	23.3	17.5	25.0	1.0	2.4	1.8	1.8	19.2	19.2	11.4	3.8	46.4
Vernal	4.6	25.3	16.9	23.3	1.0	2.3	1.7	1.9	19.1	21.4	11.5	4.0	44.0
Average	3.4	21.9	17.9	25.4	1.0	2.1	1.8	1.9	18.9	20.6	11.5	3.9	45.2

See table A1, footnote 1.
[2] See table A1, footnote 2.

TABLE A8.—*Mean squares from combined analyses of variance of coumestrol (p.p.m.) and percentage of other chemical constituents in alfalfa with regressions of coumestrol with other characters for 1960* [1]

Source of variation	d.f.	Coumestrol	Protein	Crude fiber	Ash	Fat	N-free extract
Replications in locations	7	53. 47	0. 3514	1. 7870	0. 6925**	0. 0807	2. 6817
Locations (L)	6	70, 366. 01**	154. 4147**	464. 9021**	48. 9551**	4. 3962**	173. 1808**
Regression	1		6. 1912 +	469. 5066 +	263. 4791** −	3. 2028 −	37. 6335 −
Deviations	5		184. 0594**	463. 9811**	6. 0503**	4. 6348**	200. 2902**
Varieties (V)	4	2, 582. 44*	2. 1051	2. 7900	. 5230	. 2439	2. 0214
Regression	1		3. 5876 −	2. 4329 +	. 1511 −	. 2297 −	1. 4405 +
Deviations	3		1. 6109	2. 9090	. 6470	. 2487	2. 2134
V×L	24	656. 56**	2. 7483**	7. 0417**	. 2781**	. 1373**	3. 4178
Regression	1		. 0069 −	26. 9448* +	. 0037 −	. 2863 +	20. 3564** −
Deviations	23		2. 8675**	6. 1763**	. 2900**	. 1308**	2. 6814
Error a	28	77. 59	. 3211	1. 6957	. 0972	. 0413	2. 2794
Cuttings (C)	2	19, 032. 27	15. 4002	2. 5263	9. 2508*	. 2389	6. 8807
Regression	1		1. 5261 +	2. 6139 +	17. 9889* −	. 4742 −	4. 3175 +
Deviations	1		29. 2743	2. 4387	. 5127	. 0036	9. 4439
C×L	12	8, 729. 65**	30. 4844**	59. 0339**	2. 1184**	. 7042**	59. 9748**
Regression	1		183. 2247** +	70. 9847 −	. 2649 +	. 8073 +	42. 5626 −
Deviations	11		16. 5989**	57. 9475**	2. 2869**	. 6948**	61. 5577**
C×V	8	1, 859. 65	. 6460	5. 5434	. 1146	. 0823	5. 3311
Regression	1		. 5954 +	10. 9101 +	. 0002 −	. 1541 −	13. 4619 −
Deviations	7		. 6532	4. 7767	. 1309	. 0720	4. 1695
C×V×L	48	872. 97**	1. 0942**	4. 3549	. 1706*	. 0853*	4. 4429*
Regression	1		. 7799 +	. 8422 +	. 1357 −	. 3183 −	2. 3235 −
Deviations	47		1. 1008**	4. 4296	. 1714*	. 0803*	4. 4880*
Error b	[2] 70	295. 46	. 4085	2. 8944	. 0966	. 0481	2. 6409

[1] * and ** = significant at 5- and 1-percent levels, respectively; + or − following regression mean squares indicates sign of regression coefficient.

[2] Only 54 d.f. in error for coumestrol.

TABLE A9.—*Mean squares from analyses of variance of coumestrol (p.p.m.) and percentage of other chemical constituents in alfalfa with regressions of coumestrol ("x" variable) with other characters for 1960, Davis, Calif.*[1]

Source of variation	d.f.	Coumestrol	Protein	Crude fiber	Ash	Fat	N-free extract
Replications (R)	1	7.2200	0.0184	3.9988**	0.0067	0.0519	2.4156**
Varieties (V)	4	204.6600**	6.4481**	10.2120**	.2839	.0860	1.1892**
Regression	1		3.5496 +	16.4563 −	.0000 +	.0194 +	4.1222 + *
Deviations	3		7.4143**	8.1306**	.3785	.1082	.2116*
Error a	4	10.3300	.0347	.0795	.0861	.0388	.0278
Cuttings (C)	4	1,596.6500**	9.8556**	35.0090**	4.1418**	.6172**	21.7425**
Regression	1		10.9877 +	4.5112 −	.3378 −	.2847 +	1.3067 −
Deviations	3		9.4785**	45.1750**	5.4098**	.7281**	28.5545**
C×V	16	176.3463**	1.6288**	4.6077**	.2245*	.0684	7.0701**
Regression	1		5.8203 −	.4569 −	.4053 −	.0873 −	16.1663 +
Deviations	15		1.3493*	4.8844**	.2124*	.0672	6.4637**
Error b	[2] 20	32.0874	.4397	.1978	.0832	.0528	.6072

[1] See table A8, footnote 1.
[2] 9 d.f. for coumestrol.

TABLE A10.—*Mean squares from analyses of variance of coumestrol (p.p.m.) and percentage of other chemical constituents in alfalfa with regressions of coumestrol ("x" variable) with other characters for 1960, Ames, Iowa* [1]

Source of variation	d.f.	Coumestrol	Protein	Crude fiber	Ash	Fat	N-free extract
Replications (R)	1	145. 2100	0. 7584	0. 6424	0. 1033*	0. 0065	0. 2219
Varieties (V)	4	3, 087. 4075**	. 7356	6. 6884*	. 1340*	. 1146**	4. 8661
Regression	1		. 1269−	22. 3577+ **	. 0033−	. 1618−	15. 3067− *
Deviations	3		. 9386	1. 4653	. 1775*	. 0988**	1. 3859
Error a	4	188. 4600	. 4200	. 9576	. 0126	. 0050	. 8477
Cuttings (C)	2	39, 329. 7350**	44. 3396**	79. 2052**	. 8437**	. 2144**	55. 5644**
Regression	1		88. 6789+ **	77. 4216 −	. 0003+	. 4062+ **	1. 6159 −
Deviations	1		. 0004	80. 9888**	1. 6871**	. 0225	109. 5129**
C×V	8	3, 281. 9763**	1. 2883	7. 0069*	. 1458	. 1687**	6. 7010
Regression	1		. 7357−	1. 1491+	. 0413 −	. 0208+	. 0241 −
Deviations	7		1. 3672	7. 8437*	. 1607	. 1898**	7. 6549*
Error b	[2] 10	420. 9344	. 8950	1. 9245	. 0937	. 0172	2. 2358

[1] See table A8, footnote 1.
[2] 9 d.f. for coumestrol.

TABLE A11.—*Mean squares from analyses of variance of coumestrol (p.p.m.) and percentage of other chemical constituents in alfalfa with regressions of coumestrol ("x" variable) with other characters for 1960, Manhattan, Kans.*[1]

Source of variation	d.f.	Coumestrol	Protein	Crude fiber	Ash	Fat	N-free extract
Replications (R)	1	0.3000	0.2132	3.1753**	0.1050	0.0518	5.4760*
Varieties (V)	4	494.6325*	1.9579**	8.5432**	.5618	.1381	3.1048*
Regression	1		1.8649 +	.0554 +	.0639 −	.1376 +	2.9556 −
Deviations	3		1.9889**	11.3725**	.7278*	.1387	3.1546*
Error a	4	48.7725	.0849	.1379	.0604	.0556	.3591
Cuttings (C)	3	5,992.1267**	26.0627**	21.6962**	6.4765**	.7055**	17.0617*
Regression	1		.1530 +	47.7501 + **	17.9080 − *	.4707 −	5.6808 −
Deviations	2		39.0176**	8.6693	.7608*	.8230**	22.7522*
C × V	12	229.9808**	1.1305**	4.1522	.1998	.1128*	5.8710
Regression	1		6.8822 + **	1.6819 +	.0066 +	.0508 +	17.8649 − *
Deviations	11		.6076*	4.3767	.2174	.1184*	4.7806
Error b	15	54.7780	.2072	3.0295	.1431	.0420	3.7167

[1] See table A8, footnote 1.

TABLE A12.—*Mean squares from analyses of variance of coumestrol (p.p.m.) and percentage of other chemical constituents in alfalfa with regressions of coumestrol ("x" variable) with other characters for 1960, Lincoln, Nebr.*[1]

Source of variation	d.f.	Coumestrol	Protein	Fiber	Ash	Fat	N-free extract
Replications (R)	1	91.8800	0.0182	1.2161	0.1280	0.0360	3.1883
Varieties (V)	4	995.6575**	7.8754**	13.0621	.5407	.4966**	2.5565
Regression	1	----------	12.4977−	24.4497+	.4165−	.5944−	.0000+
Deviations	3	----------	6.3346**	9.2663	.5821*	.4641**	3.4087
Error a	4	42.6350	.1646	3.4087	.0855	.0049	5.6515
Cuttings (C)	2	40.1400	5.0922**	1.9805	.7802**	1.3844**	1.5292
Regression	1	----------	3.5354−	1.6296+	.0365−	2.6034+ **	3.0081+
Deviations	1	----------	6.6489**	2.3314	1.5238**	.1655	.0502
C×V	8	403.0613*	1.0600**	.9539	.2258*	.1077	2.8454
Regression	1	----------	3.5451+	.7558+	.3686+	.0049−	13.6303− *
Deviations	7	----------	.7050**	.9822	.2054*	.1224	1.3047
Error b	10	102.1920	.1229	2.7303	.0595	.0419	2.4642

[1] See table A8, footnote 1.

TABLE A13.—*Mean squares from analyses of variance of coumestrol (p.p.m.) and percentage of other chemical constituents in alfalfa with regressions of coumestrol ("x" variable) with other characters for 1960, Raleigh, N.C.*[1]

Source of variation	d.f.	Coumestrol	Protein	Crude fiber	Ash	Fat	N-free extract
Replications (R)	1	207.0200	0.3062	0.6786	0.0189	0.2755	0.0136
Varieties (V)	4	287.4925	2.5276	1.7398	.5113*	.0489	1.1918
Regression	1		2.1943−	1.8241+	1.0193− *	.0001−	1.3177+
Deviations	3		.6388	1.7118	.3420	.0652	1.1498
Error a	4	193.5550	1.8180	2.3828	.0688	.0572	3.1470
Cuttings (C)	3	13,748.9633**	118.1009**	139.3903**	.6259**	2.5366**	11.2527**
Regression	1		143.5306+	49.0234−	.9711−	.8588+	24.2066−
Deviations	2		105.3861**	184.5738**	.4533**	3.3755**	4.7757*
C×V	12	472.0958*	.6914	1.0172	.0510	.0740	1.0354
Regression	1		.9775+	2.4982−	.0129−	.0284−	.7637+
Deviations	11		.6654	.8826	.0545	.0781	1.0600
Error b	[2] 15	150.7657	.3410	1.1706	.0284	.0346	.8403

[1] See table A8, footnote 1.
[2] 14 d.f. for coumestrol.

TABLE A14.—*Mean squares from analyses of variance of coumestrol (p.p.m.) and percentage of other chemical constituents in alfalfa with regressions of coumestrol ("x" variable) with other characters for 1960, University Park, Pa.*[1]

Source of variation	d.f.	Coumestrol	Protein	Crude fiber	Ash	Fat	N-free extract
Replications (R)	1	48. 1400	0. 2765	5. 3089*	0. 0512	0. 0554	1. 7328
Varieties (V)	4	1, 376. 8875*	. 7960	1. 4162	. 2052	. 1430	. 8979
Regression	1		. 0380+	. 1837+	. 1485+	. 0794−	. 5286−
Deviations	3		1. 0487	1. 8270	. 2241	. 1641	1. 0210
Error a	4	123. 5375	. 1784	. 2839	. 0397	. 0396	. 2981
Cuttings (C)	2	1, 148. 2650	33. 5850**	77. 8199**	9. 3462**	. 1741**	211. 7466**
Regression	1		18. 3372+	43. 8279−	5. 8831+	. 0319−	. 0083+
Deviations	1		48. 8327**	111. 8119**	12. 8094**	. 3163**	423. 4849**
C×V	8	2, 337. 5713	. 6056**	3. 5086**	. 0671	. 0828*	2. 2664*
Regression	1		1. 8515+	4. 5471+	. 0807−	. 0234+	8. 0009−**
Deviations	7		. 4277**	3. 3603**	. 0651	. 0913*	1. 4471
Error b	10	858. 0430	. 0439	. 6024	. 0776	. 0210	. 4755

[1] See table A8, footnote 1.

TABLE A15.—*Mean squares from analyses of variance of percentage of chemical constituents in alfalfa for 1960, Logan, Utah*[1]

Source of variation	d.f.	Coumestrol[2]	Protein	Crude fiber	Ash	Fat	N-free extract
Replications (R)	1	0.0500	0.0203	0.0059	4.4314*	0.0219	5.3763
Varieties (V)	4	.6688	.5416**	10.0947	.2970	.1293	7.4344
Error a	4	.7062	.0776	3.0124	.3648	.1666	5.6580
Cuttings (C)	2	68.4500**	20.1946**	115.1302**	.3174	1.2732**	51.2404*
C×V	8	.6688	.6735	9.8732	.1475	.0592	8.9493
Error b	10	.5750	.7600	10.4582	.1728	.1625	7.3443

[1] * and **=significant at 5- and 1-percent levels, respectively.

[2] Third cutting omitted in analysis of variance and regression analysis omitted because 13 samples were lost in shipment. Degrees of freedom were as follows: Cuttings 1, cuttings×varieties 4, error b 5.

TABLE A16.—*Mean squares from combined analyses of variance of coumestrol content (p.p.m.), height (inches), and defoliation with regressions of coumestrol ("x" variable) with height and defoliation, all locations except Iowa* [1]

Source of variation	d.f.	Coumestrol	Height	Defoliation	Source of variance	d.f.	Coumestrol	Height	Defoliation
Years (Y)	1	2, 565	4, 162**	33. 0	C×L	10	21, 948	386	23. 1
Locations (L)	5	115, 278*	1, 692**	58. 6**	Regression	1		1, 708+ *	6. 1+
Regression	1		1, 101—	238. 4+ *	Deviations	9		239	25. 0
Deviations	4		1, 840**	13. 6**	C×L	2	143, 801*	84	84. 5**
Y×L	5	30, 884**	257**	5. 6**	Regression	1		163— **	169. 0+ **
Regression	1		474—	2. 2—	Deviations	1		4	0
Deviations	4		203**	6. 4**	C×Y×L	10	30, 418**	534**	26. 0**
Varieties (V)	4	4, 001*	122*	7. 0*	Regression	1		553—	105. 8+ *
Regression	1		119—	27. 7+ **	Deviations	9		531**	17. 1**
Deviations	3		123*	. 1	C×V	8	1, 798	16*	. 9
V×L	20	1, 150	19	1. 8	Regression	1		42— *	. 7+
Regression	1		49+*	11. 0+ *	Deviations	7		13	. 9
Deviations	19		18	1. 3	C×V×L	40	1, 285	10	. 8
V×Y	4	452	20	1. 5	Regression	1		68— **	1. 5+
Regression	1		2+	4. 6+ *	Deviations	39		9	. 8
Deviations	3		26	. 5	C×V×Y	8	752	4	1. 1
V×Y×L	20	1, 120**	11*	1. 0**	Regression	1		2+	3. 3+ *
Regression	1		3—	0.	Deviations	7		4	. 8
Deviations	19		11*	1. 0**	C×V×Y×L	40	990**	6	. 6**
Cuttings (C)	2	74, 235	275	64. 0	Regression	1		5+	1. 5+
Regression	1		550+**	36. 3+	Deviations	39		6	. 6**
Deviations	1		0	91. 7	Error	333	435	7	. 2

[1] * and **=significant at 5- and 1-percent levels, respectively; + or — following regression mean squares indicates sign of regression coefficient.

TABLE A17.—*Mean squares from analyses of variance of coumestrol content (p.p.m.) and height (inches) with regressions of coumestrol ("x" variable) with height, Nebraska, Pennsylvania, and Utah (3 cuttings included in analyses of variance)* [1]

Source of variation	d.f.	Nebraska		Pennsylvania		Utah	
		Coumestrol	Height	Coumestrol	Height	Coumestrol	Height
Years (Y)	1	5, 866	940. 00**	1, 483	1, 216. 00**	10, 305**	1, 703. 00**
Varieties (V)	4	3, 925	11. 00	1, 986	41. 50	70	32. 75**
Regression	1		2. 97+		98. 35−		32. 35−
Deviations	3		13. 68		22. 55		32. 88**
Y×V	4	1, 106**	4. 50*	2, 072	9. 00**	43	4. 25
Regression	1		13. 91+ **		3. 95+		. 15−
Deviations	3		1. 36		10. 68**		5. 62
Cuttings (C)	2	22, 167	611. 00	30, 450	1, 058. 50	6, 256	65. 00
Regression	1		977. 49+		2, 062. 31+		55. 73−
Deviations	1		244. 51		54. 69		74. 27
C×Y	2	18, 584**	130. 50**	13, 688**	714. 50**	5, 479**	453. 00**
Regression	1		259. 39+ **		246. 93+		65. 31+
Deviations	1		1. 61		1, 182. 07**		840. 69**
C×V	8	1, 320	27. 62	3, 908	9. 00	59	3. 00
Regression	1		104. 57− *		30. 66+		15. 31−
Deviations	7		16. 63		5. 91		1. 24
C×Y×V	8	1, 172**	11. 12**	2, 313	6. 00	32	2. 62
Regression	1		. 45 −		7. 48+		3. 78+
Deviations	7		12. 65**		5. 79		2. 46
Error	[2] 60	132	1. 78	1, 596	4. 77	38	4. 77

[1] See table A16, footnote 1.
[2] 44 d.f. for coumestrol in Utah.

TABLE A18.—*Mean squares from analyses of variance of coumestrol content (p.p.m.) and height (inches) with regressions of coumestrol ("x" variable) with height, California, Kansas, and North Carolina (4 cuttings included in analyses of variance)*[1]

Source of variation	d.f.	California		Kansas		North Carolina	
		Coumestrol	Height	Coumestrol	Height	Coumestrol	Height
Years (Y)	1	1, 140*	32. 00	81, 361**	86. 00**	15, 602**	1, 398. 00**
Varieties (V)	4	276	57. 50	2, 391	22. 25	754	68. 00**
Regression	1		110. 43 −		1. 36 −		31. 27 +
Deviations	3		39. 84		29. 21		80. 24**
Y×V	4	111*	57. 25	2, 156**	15. 25**	368	6. 00
Regression	1		90. 09 −		14. 61 −		5. 76 −
Deviations	3		46. 30		15. 46**		6. 08
Cuttings (C)	3	1, 679	42. 33	82, 622	140. 33	10, 441	258. 67
Regression	1		88. 59 −		399. 42 +		418. 61 +
Deviations	2		19. 20		10. 79		178. 70
C×Y	3	2, 911**	421. 00**	164, 182**	219. 00**	19, 298**	829. 67**
Regression	1		867. 33 −		594. 36 − *		2, 476. 99 − **
Deviations	2		197. 84**		31. 32**		6. 00
C×V	12	185*	28. 50 −	1, 398	3. 00	798	7. 92
Regression	1		12. 09 +		. 18 +		6. 58 −
Deviations	11		29. 99		3. 26		8. 04
C×Y×V	12	68*	23. 50	1, 058**	2. 83**	461**	7. 58
Regression	1		35. 50 +		. 00 −		1. 84 +
Deviations	11		22. 41		3. 09**		8. 10
Error	[2] 90	36	17. 12	362	. 70	166	4. 88

[1] See table A16, footnote 1.
[2] 79 d.f. in California for coumestrol.

TABLE A19.—*Mean squares from analyses of variance of coumestrol content (p.p.m.) and height (inches) with regressions of coumestrol ("x" variable) with height, Ames, Iowa, 1961* [1]

Source of variation	d.f.	Coumestrol	Height
Varieties (V)	4	4,764**	53.75**
Regression	1		14.58—
Deviations	3		66.81**
V×R	12	1,046	2.50
Regression	1		5.76—
Deviations	11		2.20
Cuttings (C)	2	126,751**	31.50
Regression	1		11.86+
Deviations	1		51.14*
C×V	8	7,836**	8.87**
Regression	1		39.32—*
Deviations	7		4.53**
Error	30	845	.60

[1] See table A16, footnote 1.

TABLE A20.—*Variety and location mean squares from analyses of variance of defoliation with regressions of coumestrol ("x" variable) with defoliation, 1960 and 1961* [1]

Source	d.f.	California	Kansas	Nebraska	North Carolina	Pennsylvania	Utah	Locations combined
Varieties	4	0. 25	2. 75**	4. 50**	5. 50**	5. 75	1. 25	7. 00*
Regression	1	0	7. 62+	14. 74+ *	14. 63+	18. 56+	. 03−	27. 72+ **
Deviations	3	. 33	1. 13**	1. 09**	2. 46*	1. 48	1. 66	. 09
Locations	5							58. 60**
Regression	1							238. 43+ *
Deviations	4							13. 64**

[1] See table A16, footnote 1.

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